THE UNIVERSITY OF ALBERTA

THE EFFECT OF MALIGNANT DISEASE ON HUMAN AND ON RAT PLASMA GLYCOPROTEINS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

by

Julius George Bekesi

EDMONTON, ALBERTA

APRIL, [redacted] 1964

ABSTRACT

The present investigation was undertaken to study the effect of malignant disease on human and on rat plasma glycoproteins.

The levels of galactose and mannose in plasma glycoproteins, in the absence of the seromucoid fraction, were determined in 654 human subjects. This group consisted of healthy individuals as well as patients suffering from malignant and non-malignant disease. In 118 representative patients the analysis was extended to include individual determinations of hexoses (galactose-mannose), hexosamines (glucosamine-galactosamine), N-acetylneuraminic acid and fucose in the plasma glycoproteins.

Levels of carbohydrate in the plasma glycoproteins of patients with malignant disease showed significant elevation as compared with levels observed in normal individuals and patients with non-malignant disease.

The behaviour of the various protein-bound carbohydrates in patients with localized carcinoma of the breast differed from that observed in patients with other types of malignancy or suffering from carcinoma of the breast with metastases.

In ten patients with malignant disease, serial quantitative determinations of plasma glycoproteins indicated that these protein-carbohydrate complexes are intimately related to the neoplastic process.

In order to determine the relationship between the plasma glycoproteins and tumour growth, adult Sprague-Dawley rats were implanted

with Walker-256 carcinoma and serial determinations of the plasma protein-bound carbohydrates carried out. A total of 95 rats formed the test group and the animals were sacrificed at intervals following intramuscular implantation of the tumour. A control group of 32 rats was sacrificed at corresponding intervals following intramuscular injection of an homologous muscle homogenate.

Changes in the levels of galactose-mannose, glucosamine-galactosamine, N-acetylneuraminic acid and fucose of the total plasma glycoproteins, as well as the hemoglobin, hematocrit and total plasma protein were determined.

It was observed that the growth of Walker-256 carcinoma in the rat following implantation is associated with significant increases in the carbohydrate constituents of the plasma glycoproteins. However, the interval between implantation and the occurrence of a significant increase is different for each carbohydrate, and the magnitude of the increase is likewise not uniform. These observations would suggest that the site or the rate of synthesis of the various carbohydrate moieties of the plasma glycoproteins may not be similar, as is commonly believed.

ACKNOWLEDGEMENTS

The writer gratefully acknowledges the guidance and invaluable assistance of Dr. J. Tuba, under whose supervision this investigation was carried out. The author wishes to express his sincere thanks to Dr. R.A. Macbeth for interest, encouragement and helpful criticism throughout the course of this investigation. The interest of Dr. J.S. Colter and other members of the Academic Staff of the Department of Biochemistry is sincerely appreciated.

Acknowledgement is also due to Mr. R. Clelland for his assistance with the animals and to the members of Surgical-Medical Research Institute.

The skillful technical assistance of Mrs. P. Innes is gratefully acknowledged.

This investigation was supported by grants from the National Cancer Institute of Canada No. 226 and Medical Research No. 208.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABBREVIATIONS

ATP - Adenosine triphosphate

ADP - Adenosine diphosphate

GTP - Guanosine triphosphate

GDP - Guanosine diphosphate

CTP - Cytidine triphosphate

CMP - Cytidine monophosphate

UTP - Uridine triphosphate

UDP - Uridine diphosphate

UDPG - Uridine diphosphate-D-glucose

UMP - Uridine monophosphate

PP - Pyrophosphate

P_i - Inorganic phosphate

CoA-SH - Coenzyme-A

SD - Standard deviation

SE - Standard error of the mean

OD - Optical density

I. INTRODUCTION

The presence of carbohydrate in serum protein was discovered by Freund (1) in 1892, when he obtained a heat incoagulable protein fraction from human serum which differed from other known serum proteins by the significant amount of carbohydrate which was firmly bound to the protein molecule. Freund's observation was substantiated the following year when Morner (2) demonstrated the presence of increased amount of reducing sugar in acid hydrolyzates of proteins from human serum. Subsequently Zanetti (3) heated normal horse serum at 100°C, whereupon most of the serum proteins coagulated, while the new protein characterized by the presence of relatively large amount of carbohydrate, remained in the filtrate. Because of its similarity to ovomucoid, Zanetti called the protein "seromucoid".

In spite of several difficulties Zanetti (4) was able to prepare crystalline glucosamine from the acid hydrolyzate of "seromucoid". The presence of glucosamine was later confirmed by Bywaters (5). The latter showed that by mild alkaline treatment the carbohydrate moiety of seromucoid can be released in the form of a polysaccharide, precipitable by ethanol. Further work on the isolation of the individual sugars in the acid or alkaline hydrolyzate of seromucoid was prevented, at that time, by the lack of proper analytical techniques for handling the relatively small amount of individual carbohydrates present.

In 1929 Grevenstuck (6) repeated Morner's (2) experiment utilizing human plasma obtained from patients suffering from diabetes, tuberculosis and cancer. The plasma proteins were precipitated with ethanol and

hydrolyzed with hydrochloric or sulfuric acid for several hours at 120°C. The hydrolytic product which he obtained contained significant amount of reducing sugar which gave a positive reaction with the orcinol-sulfuric acid reagent similar to the reaction observed with free glucose. The concentration of the reducing sugar was many times greater in the plasma of patients suffering from these diseases than in the plasma of healthy subjects. The unknown carbohydrate, because of its similarity to glucose, was called "protein-bound glucose" by Grevenstuck.

In 1931 Lustig and Langer (7) applied the orcinol-sulfuric acid color reaction to the precipitated plasma proteins without preliminary acid hydrolysis and were able to estimate the content of protein-bound carbohydrate using a Stufenphotometer. They also noted that the amount of protein-bound hexose was greater in cancer patients than in the normal individual.

By this time it was generally accepted that the reducing sugar content of the plasma protein was increased by acid hydrolysis, but the hypothesis that the increase was the result of the liberation of protein-bound glucose was the subject of much controversy.

The next significant contribution to the understanding of the plasma carbohydrate-protein complex came from the studies of Rimington (8) who isolated and identified a dimannose-glucosamine complex from this source. In 1933 he reported further (9) that human plasma protein contains three protein-bound carbohydrates: D-galactose, D-mannose and D-glucosamine and that it had been impossible for him to demonstrate glucose in this carbohydrate-protein complex. Unfortunately Rimington's observations

attracted little attention since the interest of active workers in the field were, at that time, diverted to a new area of investigation ushered in by the demonstration of the presence of a group of acidic mucopolysaccharides which were widely distributed in body connective tissues. Thus, after a period of relatively intense investigation, the subject of the carbohydrate-protein complex of plasma lay virtually abandoned for the next twenty years.

The introduction of paper chromatography by Partridge (10, 11) for the separation and identification of small amounts of carbohydrates in 1946 reopened the investigation of the composition of the plasma carbohydrate-proteins complex.

Protein-bound fucose in human plasma proteins was demonstrated in 1948 by Dische and Shettles (12) using the cysteine-sulfuric acid color reaction. Their observation was confirmed by Waldron (13) and Winzler (14) who were able to isolate L-fucose from human plasma proteins using Partridge's (10, 11) chromatographic method.

In 1955 Odin (15) demonstrated the presence of N-acetylneuraminic acid in normal human plasma proteins by isolation, paper chromatography and colorimetric methods.

Identification of galactosamine in human plasma proteins was first achieved in 1955 by Goa (16) using chromatographic method. He also demonstrated that this new member of the protein-bound carbohydrate complex in human plasma proteins is a minor constituent.

In 1955 Winzler (14) summarized the situation and stated that six different carbohydrates had been isolated and identified in normal

human plasma proteins, namely: D-galactose, D-mannose, D-glucosamine, D-galactosamine, N-acetylneuraminic acid and L-fucose.

The rapid advances made in biochemical technology at this time enabled Schultze (17, 18, 19) to carry out comprehensive studies on normal and pathological human plasma. He was able to separate many well defined protein-carbohydrate complexes by using ethanol fractionation, paper and immunoelectrophoresis and chromatographic methods. Further, he estimated the individual carbohydrate constituent of each of the protein fractions of normal human plasma and concluded that the α_1 and α_2-globulins of human plasma are the richest in bound carbohydrates. On the other hand, he found the albumin fraction of plasma to contain very small amounts of protein-bound sugars.

Comprehensive quantitative and qualitative studies have been reported in the last five years (20, 21, 22, 23, 24, 25) dealing with the properties of the newly discovered glycoproteins, but attempts to demonstrate the site of formation of plasma glycoproteins were fruitless.

Bostrom et al (26) and Spiro (27) studied the origin of plasma glycoproteins in rabbits and rats using radioactive glucose as a tracer. The experimental results which they obtained from these studies indicated that most of the protein-bound glucosamine of normal plasma is synthesized by the liver. It is unfortunate however that there is no information available concerning the site of formation of other carbohydrate constituents of normal plasma proteins.

Significant elevation of the plasma protein-bound hexose, (ie galactose and mannose) and the plasma protein-bound hexosamine (ie glucosamine and

galactosamine) has been shown to be associated with human neoplastic disease (6, 7, 14, 22, 25, 28-35), with tuberculosis (36, 37), with diabetes complicated by vascular disease (38), and with rheumatic fever and rheumatoid arthritis (35, 39, 40). However, the magnitude of the increase in these carbohydrates was more pronounced in those patients with cancer than it was in those suffering from the other diseases mentioned.

A study on the N-acetylneuraminic acid content of human plasma in normal individuals and patients with various disease states was reported by Bohm (41). He found an increase in the plasma protein-bound N-acetylneuraminic acid content in many diseases, such as pneumonia, active tuberculosis and advanced carcinoma. Further, he studied the distribution of N-acetylneuraminic acid in various human plasma protein fractions and found that the α_1 and α_2-globulins are the richest source of this carbohydrate. Bohm (41) and Saifer (42, 43) also observed that the N-acetylneuraminic acid content of the various globulin fractions did not vary directly with quantitative changes in these fractions. Increases, for example, were most marked in the carbohydrate rich α_2-globulin fraction while decreases were apparent in the γ-globulin fraction.

There is little information available concerning the plasma protein-bound carbohydrates in laboratory animals bearing transplantable tumours. Those papers that have appeared have been concerned only with the estimation of the plasma hexoses (galactose and mannose) without estimation of the plasma hexosamines, fucose or N-acetylneuraminic acid. In most of

the publications the protein-bound hexose levels were estimated either in total plasma protein alone (44, 45) or in combination with the seromucoid fraction (46, 47). Rats of the Sprague-Dawley (45, 47) and Wistar (46) strains, as well as Swiss and C_3H mice (44) have served as the host system and the tumour implants have included Walker-256 carcinoma (45, 47), sarcoma-66 (46) and mouse fibrosarcoma (44). The site of implantation has also been variable, - in one series subdermal (45), in another series subcutaneous (44, 46) and in yet another both subcutaneous and intramuscular (47) implantation was favored. This latter report demonstrated quantitative differences in the serum values of rats bearing subcutaneous as opposed to intramuscular implants and emphasized the influence of the tumour site in relation to its systemic effects.

In all of these animal studies, elevation of the hexoses, of varying magnitude, was observed in the total plasma protein and in the seromucoid fraction, once tumour growth was well established.

Numerous theories have been put forward in an attempt to explain the elevated plasma glycoprotein levels observed in various disease states, but none of these can be accepted as being adequately substantiated. It is suggested (17, 18, 33, 48, 49) that the increase observed in malignant disease is to be accounted for mainly as a result of increase in the carbohydrate bound to the plasma α_2-globulin fraction. Catchpole (44) working with experimental tumours, postulated that the increase in plasma glycoproteins arises as a result of depolymerization of the ground substance of the connective tissue adjacent to the tumour with subsequent release of these compounds into the circulation. Shetlar (35, 40) suggested that

the malignant tumour initiates a reaction in the surrounding tissue which causes increased production of the plasma glycoproteins and that these glycoproteins may have some physiological importance in the bodies defence against malignant disease. The demonstration by Dische and Osnos (50) that various animal tissues are, in fact, composed of neutral glycoproteins containing the same carbohydrate constituents as those found in plasma, would tend to lend support to the possibility that increases local production and direct release into the circulation can occur.

Seibert et al (34) have suggested that the elevation of the plasma glycoproteins merely reflect the occurrence of tissue destruction associated with tumour growth. In any case, while there is no unanimity of opinion as to the source of the glycoprotein there is little doubt, from the reports of Winzler (14), Shetlar (40), Bonomo and Bonnelli (51) and Mecroff (52), that growth of malignant human tumours is associated with increases in the plasma glycoprotein levels.

Studies reported to date, whether on human plasma or the plasma of experimental animals bearing transplanted tumours, have restricted themselves to an investigation of only one of the protein-bound carbohydrates and comparative data on synchronous changes in the various carbohydrates is not available. This information would obviously be of considerable importance in clarifying whether the various protein-bound carbohydrates have a common origin and whether their reaction to the presence of disease is quantitatively and temporally identical. The experimental work to be reported was designed to clarify these existing deficiencies in our present knowledge.

In essence the experimental design was four-fold in nature,

1/ To confirm the reported variation in the level of plasma protein-bound hexoses in a large series of normal humans and patients suffering from a wide spectrum of non-malignant diseases, and on the other hand patients with proven malignant disease.

2/ To investigate the comparative levels of all known plasma protein-bound carbohydrates (hexoses, hexosamines, N-acetylneuraminic acid and fucose) in a select sample of the above groups.

3/ To follow serially the various protein-bound carbohydrates in the plasma of humans suffering from malignant disease in response to various modalities of treatment.

4/ To follow, in the experimental animal, since human studies in this regard were obviously impossible, the changes in the plasma levels of all known protein-bound carbohydrate prior to and during the establishment and growth of implanted malignant tumours.

II. CLASSIFICATION AND CHEMICAL LINKAGE OF PLASMA GLYCOPROTEINS

Meyer (53) has recently suggested that glycoproteins may be of two general types: "one having the protein bonded to carbohydrate through polar linkages which are relatively easily split by concentrated salt, or by alkaline solutions, and the other with firmer, presumably covalent, bonds between the protein and carbohydrate". Meyer (53) proposed that the former type be designated as mucopolysaccharides like hyaluronic acid, chondroitin sulfate, keratosulfate and heparin. The second type of glycoprotein, in which the carbohydrate is more firmly bound to the protein moiety, be designated as plasma or tissue glycoprotein. This latter protein-carbohydrate complex will be discussed in some detail.

All the major plasma protein fractions have been found to contain carbohydrate prosthetic groups. In normal human blood plasma protein-bound carbohydrate is about 3 times greater in concentration than free glucose and this may significantly increase in certain pathological states.

There has been no generally accepted terminology dealing with carbohydrate containing proteins of plasma. There are many terms used to describe these conjugated proteins, e.g. mucoprotein, protein-bound carbohydrate, polysaccharide and glycoprotein, and frequently authors used the same term to designate substances of different character. However, there is a general tendency now to accept the recommendation of "The Committee on Protein Nomenclature of the American Physiological Society" and "The American Society of Biochemists" (54): "that compounds

of the protein molecule with a substance or substances containing a carbohydrate group, other than nucleic acid, be classified as glycoproteins". This classification was adopted in our laboratory, since the name of "plasma glycoproteins" more accurately indicates the nature of the components than does, for example "plasma polysaccharide" or "mucoprotein".

Plasma proteins contain many well defined specific glycoprotein fractions: seromucoids, orosomucoid, α_2-seromucoids, ceruloplasmin (transporter of copper), haptoglobin (transporter of hemoglobin in the plasma), α_2-macroglobulins, etc. However, only the first three glycoproteins will be discussed here in some detail, because of their direct contributions to the abnormally elevated plasma glycoproteins.

Seromucoid: a plasma protein fraction which is soluble in 0.75 M perchloric acid and can be precipitated by ethanol or phosphotungstic acid (2, 55). This fraction contains about 1% of the total plasma protein and 10% of the total plasma carbohydrates. However, it was demonstrated that this glycoprotein is not homogeneous because it can be further separated into two well defined glycoprotein fractions, namely: orosomucoid and α_2-seromucoid.

Orosomucoid: (acidic α_1-seromucoid) has been isolated from human plasma by saturation with ammonium sulfate at pH 3.7 or from Cohn's fractions VI and VII. It may be purified by precipitation with ethanol at pH 5.8 and at -5°C in the presence of barium or zinc ions (56, 57, 58). The molecular weight of 41,000-44,000 is relatively small, but about 41% of this is carbohydrate.

α_2-seromucoid: a group of glycoproteins of high carbohydrate content have been isolated from the supernatant fluid of Cohn's fraction V of human plasma. (59). Centrifugal analysis as well as electrophoretic treatment at pH 8.6 of this protein-carbohydrate complex resulted no further fractionation. However, after electrophoresis at pH 3.5-4.0, three major components can be identified.

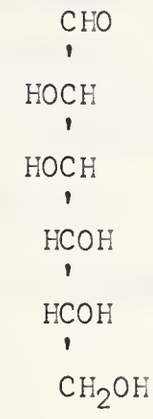
The monosaccharides in plasma glycoproteins are firmly bound to protein and cannot be separated without drastic treatment with alkali, or acid, at high temperature. Great interest has been manifested in the type of chemical bonds between the carbohydrate prosthetic group and the apo-protein. Most of the information in this regard has originated from the work of Masamune (60) and Gottschalk (61). The former studied blood group glycoproteins and suggested that there are two carbohydrate-protein bonds: a/ the hydroxyl group of serine in the polypeptide chain forms an O-glycosidic bond with the reducing group of N-acetyl-glucosamine or galactose and b/ an N-glycosidic linkage between the free amino groups of a terminal aspartic acid and the reducing group of N-acetyl-glucosamine.

Gottschalk (61) working with ovine submaxillary gland glycoprotein, concluded that the linkage of the prosthetic group of the polypeptide was a glycosidic-ester type, which involves the reducing group of an N-acetyl-hexosamine and the free carboxyl group of aspartic or glutamic acid. Further it was shown (61) that N-acetylneuraminic acid has a terminal position in the carbohydrate moiety and can be split off by neuraminidase. The enzyme showed great specificity of cleavage of the link between the

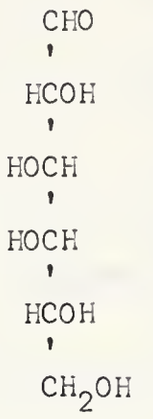
reducing group of sialic acid and a hydroxyl group of the adjacent monosaccharide.

III. THE MONOSACCHARIDE COMPONENTS OF THE PLASMA GLYCOPROTEINS AND THEIR BIOSYNTHESIS

A. Hexoses



D-Mannose



D-Galactose

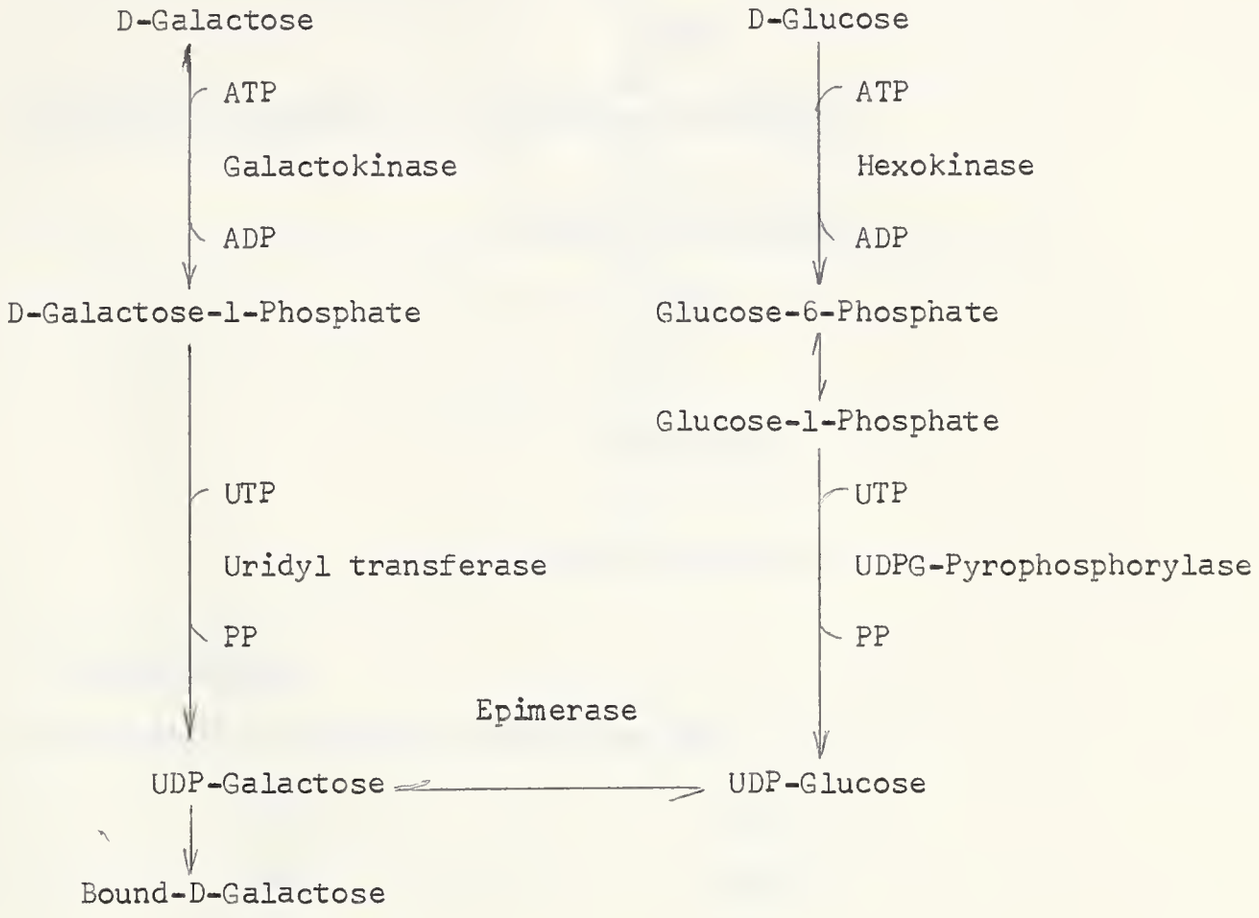
The presence of D-mannose in plasma protein was first demonstrated by Dische in 1928 (62). Two years later Bierry (63, 64) working with albumin from human plasma was able to isolate a second carbohydrate which was shown to be D-galactose. More evidence about the exact occurrence of these sugars in the plasma glycoproteins has come from the paper chromatographic studies of Winzler (14), who showed that the plasma glycoproteins contain D-galactose and D-mannose in approximately equal amount.

Biosynthesis of D-galactose and D-mannose

Recent information indicates that the sugar nucleotides are of prime importance in the biosynthesis of tissue and plasma glycoprotein carbohydrate.

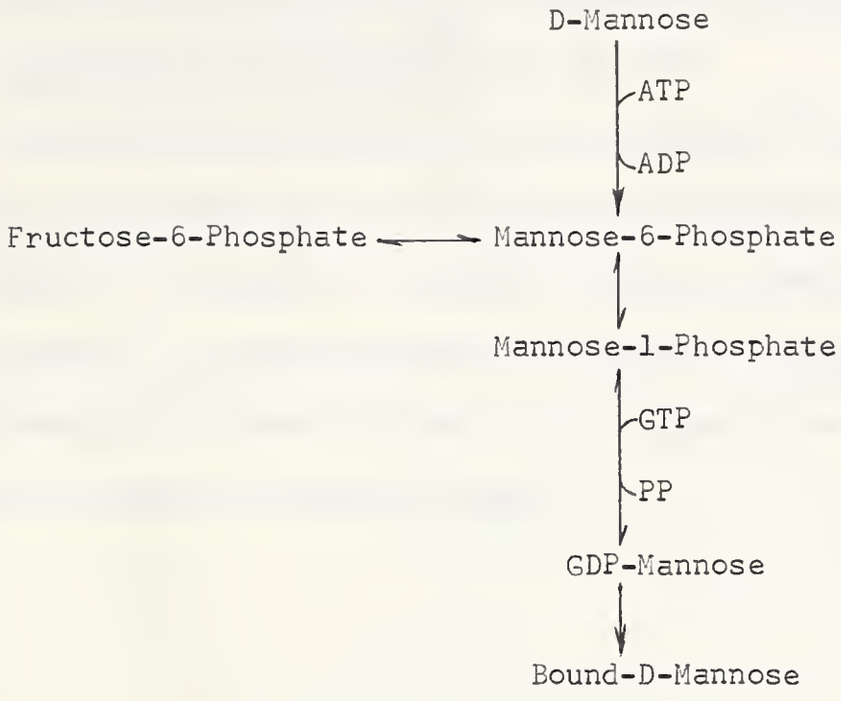
The metabolism of D-galactose has recently been reviewed (65, 66) and it was shown that uridine triphosphate is the sugar nucleotide which is involved in the biosynthesis of D-galactose.

The following metabolic pathway for D-galactose has been recognized:



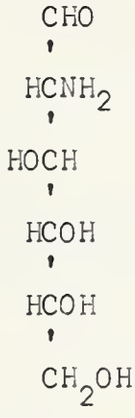
The recent isolation of guanosine diphospho-mannose by Cabib and Leloir (67) would suggest that this intermediate form may be an essential link in the incorporation of D-mannose into the glycoprotein complex.

The following metabolic pathway for D-mannose has been recognized:



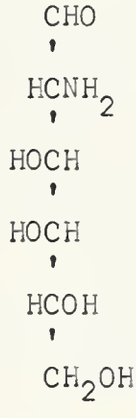
B. Hexosamines

The commonly occurring hexosamines are:



D-Glucosamine

(2-amino-2-deoxyglucose)



D-Galactosamine

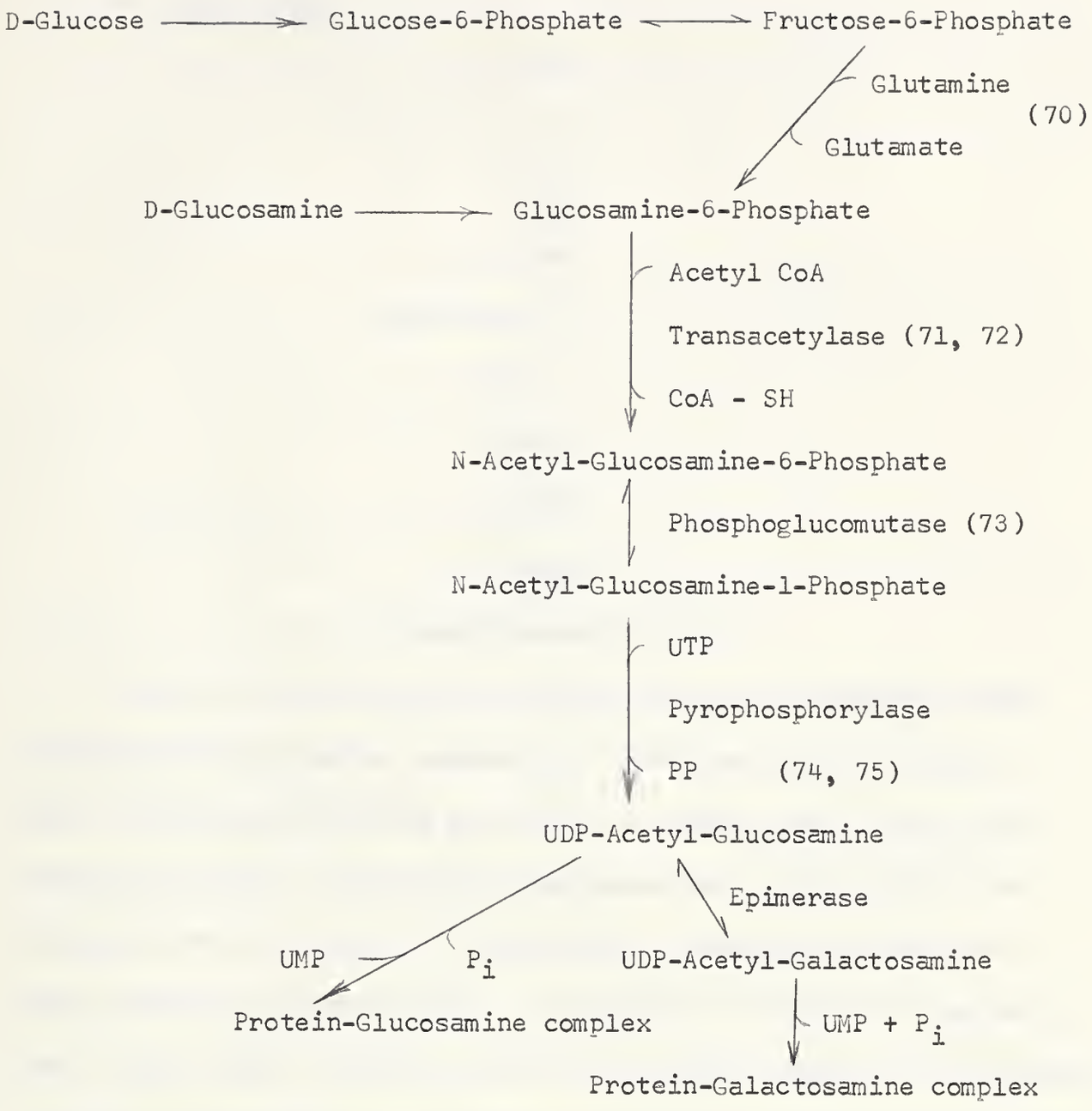
(2-amino-2-deoxygalactose)

These monosaccharides have been found among the hydrolytic products of animal tissue mucoproteins, plasma glycoproteins and bacterial polysaccharides. D-glucosamine is also a constituent of hyaluronic acid, keratosulfate, heparin and chitin. Sulfated D-galactosamine is part

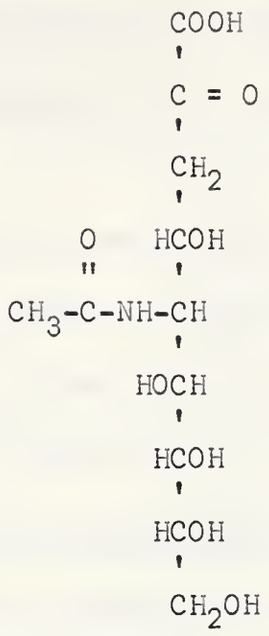
of the repeating structural unit of chondroitin sulfates.

Biosynthesis of hexosamines (27, 68, 69)

All carbohydrates found in plasma glycoprotein can arise from glucose. It was shown by Spiro (27), Shetlar (68) and Roseman (69) that labeled C^{14} glucose was converted without cleavage to glucosamine. However, the transformation of glucose to acetylated glucosamine, which is the form in which it is present in the glycoproteins, requires at least six metabolic steps:


D-Glucose
Glucose-6-Phosphate
Fructose-6-Phosphate
Glutamine
Glutamate
(70)
D-Glucosamine
Glucosamine-6-Phosphate
Acetyl CoA
Transacetylase (71, 72)
CoA - SH
N-Acetyl-Glucosamine-6-Phosphate
Phosphoglucomutase (73)
N-Acetyl-Glucosamine-1-Phosphate
UTP
Pyrophosphorylase
PP (74, 75)
UDP-Acetyl-Glucosamine
Epimerase
UMP
P_i
UDP-Acetyl-Galactosamine
Protein-Glucosamine complex
UMP + P_i
Protein-Galactosamine complex

C. Sialic acid



N-acetylneuraminic acid

Blix (76) in 1936 first crystallized a new carbohydrate from the mucoprotein of bovine submaxillary gland and named it sialic acid. This sugar derivative was shown to contain amino, acetyl and carboxylic groups. It has since been shown that sialic acid is an integral part of a number of mucoproteins. Because more than one form of sialic acid exists Blix, Gottschalk and Klenk (77) suggested that "sialic acid" should be used as the generic name for the naturally occurring neuraminic acid compounds including N-acetylneuraminic acid, N-glycolylneuraminic acid and diacetylneuraminic acid.

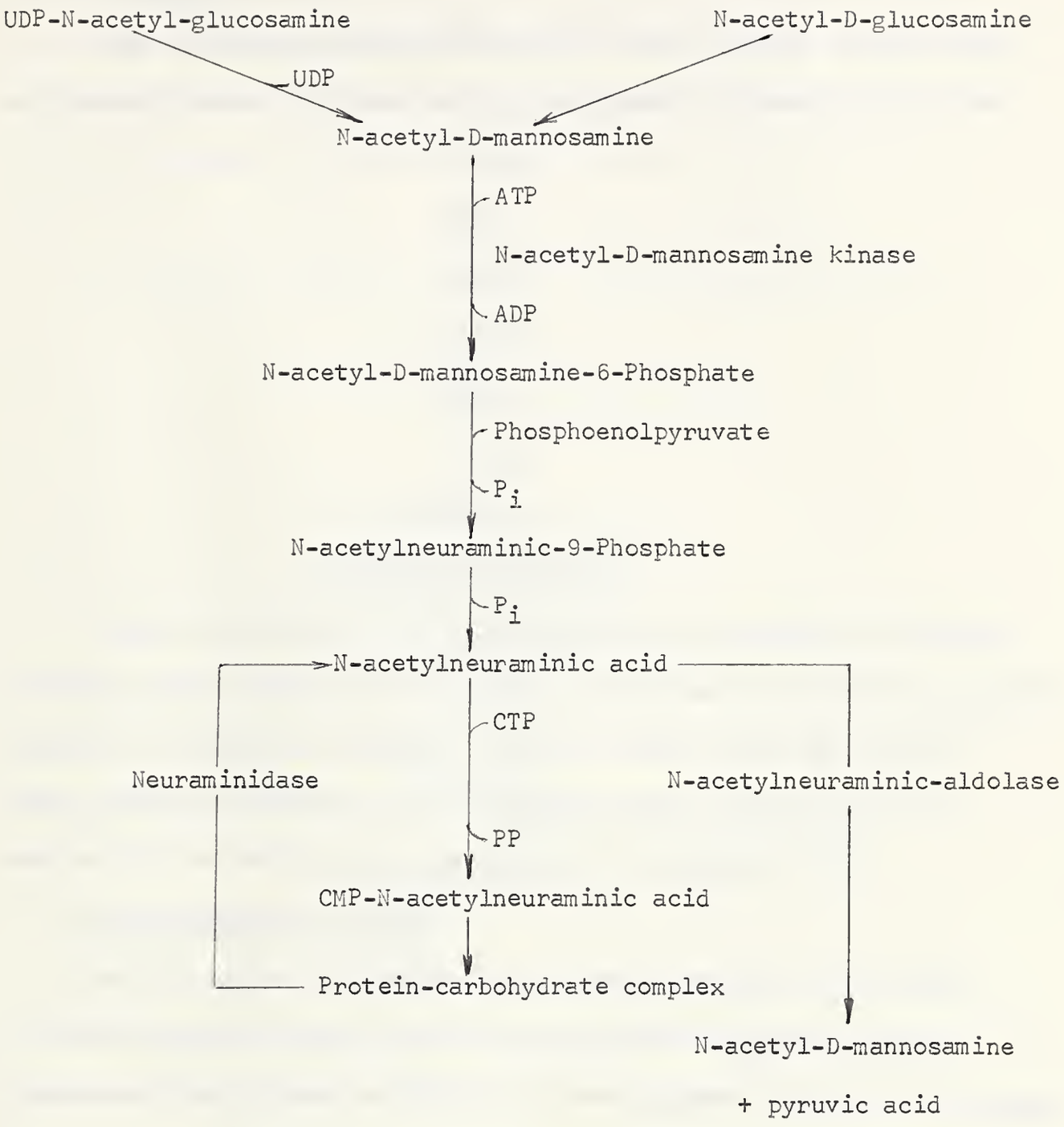
There has been considerable confusion regarding the composition of various sialic acids. It was initially considered (78) that N-acetyl-neuraminic acid was the condensation product of N-acetyl-glucosamine and pyruvic acid. However, the discovery of mammalian N-acetylneuraminic acid aldolase by Comb and Roseman (79) has demonstrated that N-acetyl-

mannosamine is the hexosamine constituent of N-acetylneuraminic acid. A recent publication by Warren and Blacklow (80) suggests that N-acetyl-neuraminic acid is synthesized from N-acetyl-mannosamine and phospho-enolpyruvic acid.

Werner and Odin (81) have shown that the sialic acid in human plasma is present as N-acetylneuraminic acid. However, there appears to be no documentation of the type of sialic acid present in the serum glycoprotein of other animal species.

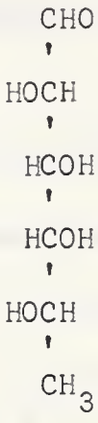
Biosynthesis of N-acetylneuraminic acid (82, 83, 84, 85, 86)

The biosynthesis of mammalian and bacterial N-acetylneuraminic acid has been established as follows:


UDP-N-acetyl-glucosamine
N-acetyl-D-glucosamine
UDP
N-acetyl-D-mannosamine
ATP
N-acetyl-D-mannosamine kinase
ADP
N-acetyl-D-mannosamine-6-Phosphate
Phosphoenolpyruvate
P_i
N-acetylneuraminic-9-Phosphate
P_i
N-acetylneuraminic acid
CTP
Neuraminidase
N-acetylneuraminic-aldolase
PP
CMP-N-acetylneuraminic acid
Protein-carbohydrate complex
N-acetyl-D-mannosamine
+ pyruvic acid

D. Fucose

This carbohydrate has been isolated from bacteria, various plant and animal tissues, and from the glycoproteins of plasma and of urine.



L-Fucose

(6-deoxy-L-galactose)

Dische and Shettles (12) first reported the presence of L-fucose in human plasma glycoproteins in 1948. It was observed that the γ-globulin fraction of plasma has a greater amount of this sugar than have the other globulin fractions. It was suggested that L-fucose might be involved in the immunological properties of γ-globulin (87).

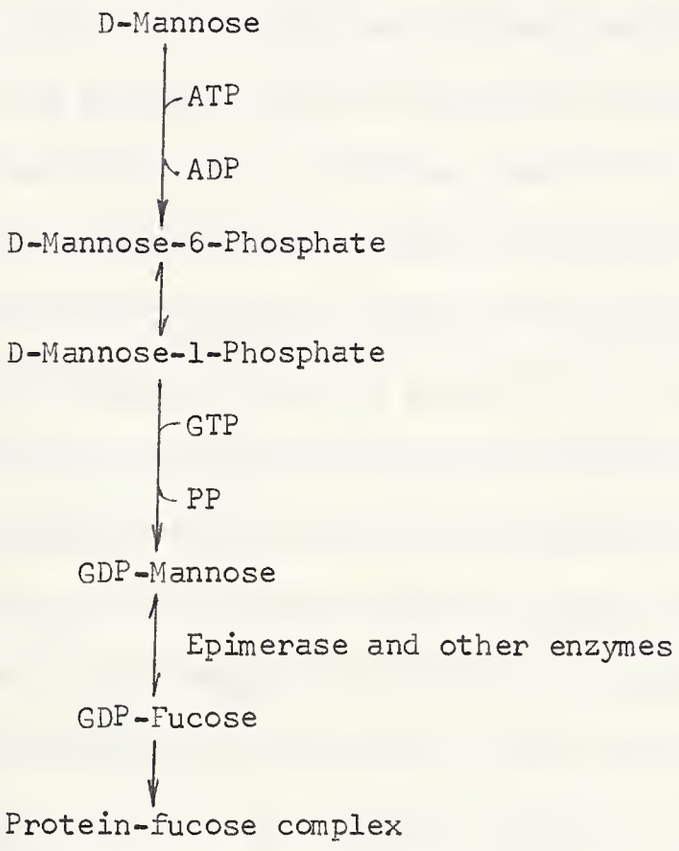
Biosynthesis of L-fucose

Early attempts to discover the synthetic pathway of this sugar have been centered largely around tracer studies utilizing labeled glucose-1-C^{14} or glucose-6-C^{14}. It was soon apparent however, that glucose could not be converted directly to L-fucose. After many fruitless experiments, Ginsburg and Kirkman (88) isolated GDP-fucose from bacteria. This was the first indication that the biosynthesis and activation of this sugar may be different from the other protein-bound carbohydrates. Ginsburg (89), continuing his work on bacteria, was able to isolate

GDP-mannose, which could be converted to GDP-fucose in the presence of an extract from Aerobacter aerogenes.

He concluded that the over all conversion of GDP-mannose to GDP-fucose involves epimerization at C-3-4 and -5 and reduction at C-6.

The suggested metabolic pathway for L-fucose is as follows:



Information concerning the metabolism of L-fucose in the mammalian organism is limited, but extensive research in this field was apparent at the recent symposium on connective tissue in New York where an identical metabolic pathway was demonstrated for L-fucose.

IV. MATERIALS AND METHODS

Blood samples were obtained from a total of 654 human subjects. One group of 101 samples was obtained from healthy adult blood donors through the courtesy of the Canadian Red Cross. An additional 518 samples were obtained from random admissions to the Surgical Service of the University of Alberta Hospital through the cooperation of the Division of Laboratory Services. Thirty-three of these samples were obtained at intervals from patients already included in the study in order to obtain follow-up information. The final group of 65 samples was made available by the Edmonton Cancer Clinic and obtained from patients suffering from proven malignant disease. The plasma of healthy blood donors constituted the control group. The clinical diagnosis in the other two groups was not known at the time the biochemical studies were carried out and only at the completion of the study were the hospital and cancer clinic records reviewed and the plasma glycoprotein levels correlated with the clinical diagnosis. All cases of malignant disease were histologically proven, but in the case of patients with non-malignant disease the discharge diagnosis was of necessity accepted whether histologically proven or not.

Samples consisted of at least 5 ml of blood collected in heparinized tubes and stored at 2°C in a cold room. All determinations were begun within four hours of obtaining the sample.

For that portion of the present study involving a transplantable animal tumour adult (2-3 months) male Sprague-Dawley rats weighing from 230 to 300 gms were used. They were housed two per cage and maintained

on a standard grain diet (90) with tap water ad libitum.

Sprague-Dawley rats bearing 5 or 6 day old Walker-256 carcinoma were selected as donors. Donors were anaesthetized with Nembutal and the entire tumour removed intact and transferred to a beaker containing sterile normal saline. Tumours were then dissected free of connective tissue and blacks of apparently viable tumour tissue were transferred to a glass homogenizer. Three parts of sterile saline were then added to the combined tumour tissue blocks and gently homogenized for 30-40 seconds. Microscopic examination of the homogenate revealed that 85-90% of the cells were intact. Implantation of 0.1 ml of this homogenate into the thigh musculature of the right hind limb of recipient rats was carried out by means of a 1 cc tuberculin syringe fitted with a 24 gauge needle. Control animals were injected with 0.15 cc of homogenized homogenous muscle, prepared and implanted in identical fashion.

Thirty-eight rats served as untreated controls, 32 animals with transplanted muscle homogenate served as treated control while 95 animals received tumour implants. No implantation failures occurred.

Test animals were sacrificed in groups, commencing 24 hours following implantation, by exsanguination from the abdominal aorta under light ether anaesthesia. The heparanized blood samples were immediately centrifuged using a Serval centrifuge at 3,500-4,000 RPM for 15 minutes. Plasma was removed and stored in a cold room at 2°C and analyses begun within 2 hours of obtaining the sample.

Hemoglobin content of the test animals blood was estimated by

the cyanomethemoglobin method as described by Hainline (91) and the hematocrit by the method of Natelson (92). The total plasma protein was determined by the modified Biuret method of Wolfson and Cohn (93) using Lab-Trol protein standard.

A. Isolation and quantitative estimation of D-galactose and D-mannose from human and rat plasma glycoprotein

In the studies on human plasma glycoprotein to be reported it was arbitrarily decided to perform the determinations in the absence of the seromucoid fraction although it was appreciated that approximately 10% of the total protein-bound hexose (galactose-mannose) of normal plasma was present in the seromucoid (14, 47). The seromucoid fraction was therefore removed by taking advantage of its known solubility in 0.75 M perchloric acid (95) before the plasma protein was subjected to colorimetric reaction with orcinol. In the studies on rat plasma the seromucoid was not removed and estimations were carried out on the total rat plasma glycoproteins.

Several colorimetric methods are available for the estimation of the protein-bound hexoses directly from the plasma protein. Each of these techniques involves the use of a strong mineral acid, most frequently sulfuric acid.

In this laboratory the modified orcinol-sulfuric acid reaction was utilized because of its simplicity and reproducability.

The orcinol-sulfuric acid reaction was initially worked out by Tillmans and Phillippi (94) for free hexoses. Lustig (7), who became interested in the presence of galactose and mannose in plasma protein,

observed that when human plasma was precipitated with ethanol, then dissolved in sodium hydroxide and the sample heated with orcinol-sulfuric acid reagent, it gave a color similar to that produced by free galactose and mannose. The original method subsequently underwent several changes. The most recent modification was that suggested by Weimer and Moshin (95), who established the optimal temperature for color development. This method has gained wide acceptance and was the one selected for use in this study.

The time for optimal color development was first reestablished. Since it is known that plasma proteins contain equal amounts of galactose and mannose the standard solution used in these preliminary studies was prepared in the same proportions, namely 1:1 w/w. The standard solution in this instance contained 200 µgms % total hexoses. Parallel studies were carried out with a representative plasma sample. The results are tabulated in Table I. It is clear from this table that the optimal color yield occurred after a 20 minute heating period at 80°C and all subsequent determinations therefore utilized a 20 minute heating period.

For the quantitative estimation of D-galactose and D-mannose in plasma glycoprotein a standard curve was obtained, using five different concentrations. Since plasma protein contains approximately equal amounts of these hexoses, the standards were made with the same proportions (1:1 w/w).

Each value presented in Table II and Figure 1 is the mean of five determinations for each concentration. The color intensity is

Table I

Relationship Between Heating Time and Maximal Color Development for Galactose-Mannose and Plasma Glycoproteins

Heating time in minutes	D-galactose and D-mannose	
	Standard #	Plasma glycoproteins *
0	0	0
5	0.035	0.019
10	0.248	0.147
12	0.298	0.224
14	0.334	0.284
16	0.350	0.321
18	0.358	0.336
20	0.386	0.360
25	0.390	0.364
30	0.393	0.368

The results are expressed in optical density

= 200 µgms of galactose-mannose in each test tube
* = 0.1 ml rat plasma in each test tube

Table II

Standardization of D-galactose and D-mannose

Readings at 540 mμ

D-galactose and D-mannose in μgms	Optical density
50	0.093
100	0.184
150	0.290
200	0.403
250	0.517

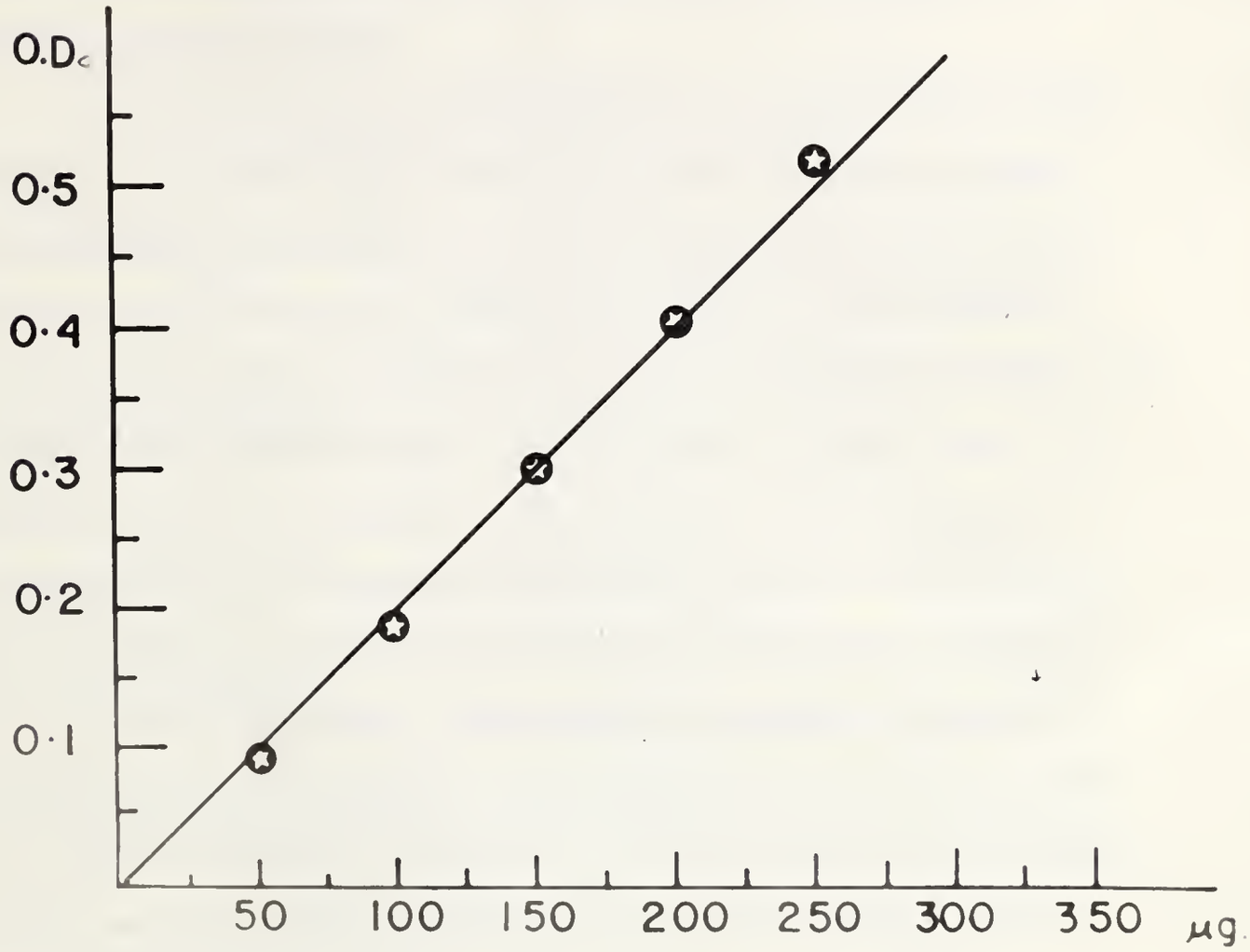


Figure 1

Relationship Between Concentration of D-galactose-D-mannose and Optical Density

shown to be proportional to the concentration of the mixture of D-galactose and D-mannose.

The reliability of the modified orcinol reaction (95) has been tested as it follows: sixty plasma samples divided into six groups of ten samples were utilized for this investigation. In the case of groups A and B 5 ml of 95% ethanol were added to 0.2 ml samples of normal human plasma, in groups C and D to 0.2 ml samples of normal rat plasma, and in groups E and F to 0.2 ml samples of plasma from rats bearing Walker-256 carcinoma. After 30 minutes the samples were centrifuged and the precipitate was washed twice with 5 ml ethanol, recentrifuged and drained. The protein precipitates were dissolved in 0.2 N sodium hydroxide. A known amount of standard D-galactose-D-mannose (1:1 w/w) mixture was then added to all samples in groups B, D and F as indicated in Table III. The total D-galactose-D-mannose content was then estimated, utilizing the modified orcinol color reaction (95).

1. Identification of plasma glycoprotein hexoses

For further identification of the type of hexoses which are present in the plasma glycoprotein, a purification and paper chromatographic analysis was necessary.

To 200 ml of human and rat plasma 800 ml of 95% ethanol was added, mixed and heated in a water bath at 80°C for 30 minutes. This precipitated the plasma proteins and after cooling and centrifuging the residue was washed twice in 400 ml of ethanol and recentrifuged. The precipitate was hydrolyzed with 300 ml of 2.0 N sulfuric acid

Table III

Recovery of D-galactose and D-mannose

Sample	Estimated D-galactose and D-mannose	% recovery of added hexoses
150 µgms of D-galactose and D-mannose mixture	150.0 ±2.42	-
A. 0.2 ml of human plasma	193.6 ±7.60	-
B. 0.2 ml of human plasma, 150 µgms of standard mixture	343.6 ±6.10	93.4
C. 0.2 ml of normal rat plasma	288.7 ±9.30	-
D. 0.2 ml of normal rat plasma, 150 µgms of standard mixture	426.4 ±10.5	91.8
E. 0.2 ml of tumour bearing rat plasma	388.2 ±8.70	-
F. 0.2 ml of tumour bearing rat plasma, 150 µgms of standard mixture	525.6 ±12.1	91.6

Results are expressed in µgm, ± standard deviation

$$\text{Standard Deviation} = \sqrt{\frac{\Sigma fx^2}{N} - \frac{(\Sigma fx)^2}{N^2}}$$

at 100°C for 2 hours in order to liberate the protein-bound hexoses. The hydrolyzate was cooled, centrifuged and the supernatant was removed. The pH of the supernatant was adjusted with saturated barium hydroxide to pH 6 and the precipitate of barium sulfate was filtered off. The filtrate was passed through a Dowex-50 cation exchange column, where the remaining inorganic ions and the free amino sugars were retained. The effluent, which contained all the liberated hexoses, was concentrated in vacuo to one fifth its original volume and lyopholized to dryness.

For identification of the hexoses the unknown sample of D-galactose-D-mannose and L-fucose standards were spotted on Whatman #1 paper using n-butanol-acetic acid-water (4:1:5) as the solvent system. After 36 hours the chromatographic paper was dried and sprayed with silver nitrate-ammonium hydroxide (1:1) reagent and dried at 100°C for 10 minutes.

The results of paper chromatographic analysis in Figure 2 indicate that normal human and rat plasma glycoprotein contain two major hexoses: D-galactose and D-mannose with a small amount of L-fucose.

2. Procedure used for routine analysis

Reagents:

1/ 95% ethanol

2/ Orcinol-sulfuric acid reagent. "A" - 60 ml of concentrated sulfuric acid and 40 ml of water, "B" - 1.6 gm of orcinol dissolved in 100 ml of water. The composition of the working reagent is 7.5 volume

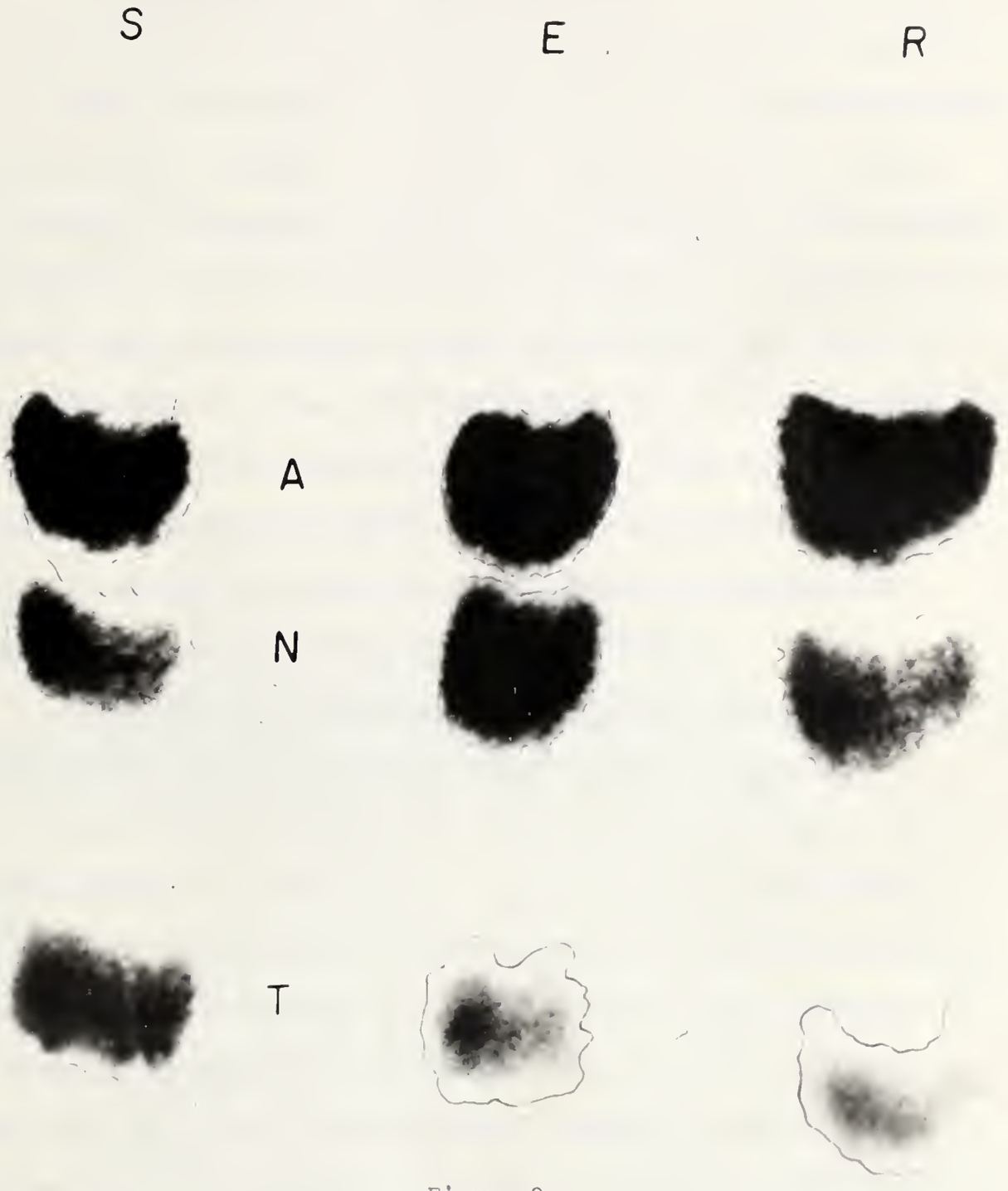


Figure 2

Paper Chromatogram of D-galactose, D-mannose, L-fucose and Hexoses from Human and Rat Plasma Glycoproteins

S = Standards
E = Hexoses from human plasma glycoproteins
R = Hexoses from rat plasma glycoproteins
A = D-galactose, N = D-mannose, T = L-fucose

of reagent "A" mixed with 1 volume of reagent "B".

3/ D-galactose and D-mannose standard

Duplicates as well as sample blanks were run on each experiment.

To each test tube 0.2 ml of plasma and 5 ml of ethanol was added, mixed and after 30 minutes, centrifuged. Samples were twice washed with ethanol. The protein precipitates were drained for 30-60 minutes, then dissolved in 1 ml of 0.2 N sodium hydroxide and transferred to an ice bath, where 8.5 ml of ice cold orcinol-sulfuric acid reagent was added and mixed. The tubes were then immersed in an 80°C water bath and heated at this temperature for 20 minutes, after which they were cooled in tap water. The reddish-pink color which developed remained stable for at least two hours at room temperature. Readings were taken on a Beckman DU Spectrophotometer at 540 mμ.

B. Isolation and quantitative estimation of D-glucosamine and D-galactosamine from human and rat plasma glycoproteins (96, 97)

In 1933 Elson and Morgan (96) reported that free amino sugars can be estimated colorimetricly. Schloss (98) made a comprehensive study of this subject, particularly regarding the chromogen formation. He reported that the number of chromogens depend largely upon the temperature used during the acetylation. Boas (97) left no doubt about the importance of the acetylation temperature and the pH requirement for the above reaction and proved that the modified Elson and Morgan (96) colorimetric method can be applied to the quantitative estimation of both amino sugars (glucosamine and galactosamine).

proteins, Boas' (97) modified technique was used.

The colorimetric method of Elson and Morgan can be applied only to free amino sugars, consequently it is necessary to liberate the sugar moiety from the conjugated protein before it can be estimated colorimetrically.

The first step was therefore to find a suitable acid concentration for hydrolysis of glycoproteins without affecting the structure of the hexosamines. For this purpose 10 mgms of standard D-glucosamine was weighed into each of 53 reaction flasks and 2 ml of hydrochloric acid was added (the concentration of hydrochloric acid in each flask is indicated in Table IV). The amino sugar-acid mixtures were then heated at 100°C in a sand bath for either 5 hours or 20 hours and the residual D-glucosamine estimated colorimetrically.

The results recorded in Table IV indicate that there is no significant destruction of D-glucosamine by hydrochloric acid as strong as 4.0 N when the reaction time was 5 hours. However, when the acid concentration is higher than 4.0 N, decomposition of the amino sugar becomes marked even with a heating time of only 5 hours.

The time requirement for the liberation of hexosamines from plasma glycoprotein was next investigated. Samples of 0.2 ml of normal rat plasma were first precipitated with 95% ethanol, mixed and centrifuged. They were then washed twice with ethanol and drained for 30-60 minutes by inversion. To each bottle 2 ml of 3.0 N hydrochloric acid was added and the mixture hydrolyzed for various length of time in a sand bath at 100°C.

Table IV and V demonstrate that hydrolysis with 3.0 N hydrochloric

Table IV

The Effect of Various Concentration of Hydrochloric Acid on D-glucosamine

No. of experiments	Normality of HCl	Heating time in hours	D-glucosamine initial	D-glucosamine after heating	Recovery in %
5	0	0	10.0		100.0
4	1	5	10.0	9.82 ±0.05	98.2
4	1	20	10.0	9.76 ±0.06	97.6
4	2	5	10.0	9.75 ±0.03	97.5
4	2	20	10.0	9.63 ±0.07	96.3
4	3	5	10.0	9.74 ±0.04	97.4
4	3	20	10.0	9.54 ±0.04	95.4
4	4	5	10.0	9.41 ±0.05	94.1
4	4	20	10.0	8.93 ±0.02	89.3
4	5	5	10.0	7.92 ±0.02	79.2
4	5	20	10.0	7.01 ±0.08	70.1
4	6	5	10.0	6.95 ±0.09	69.5
4	6	20	10.0	6.50 ±0.05	65.0

Results are expressed in mgms, ± standard deviation

Table V

Liberation of Hexosamines from Plasma Glycoproteins

Hydrolysis time in hours	Free hexosamines in mgm%
0.5	50.7 ±2.8
1.0	70.0 ±1.1
1.5	87.4 ±1.3
2.0	101.0 ±2.3
2.5	106.2 ±1.9
3.0	109.0 ±4.1
3.5	110.0 ±1.5
4.0	106.2 ±0.9
4.5	103.5 ±1.6
5.0	103.5 ±0.9
5.5	98.0 ±4.9
6.0	95.0 ±3.6

Results are expressed as mgm hexosamine per 100 ml of plasma, ± standard deviation

acid for 3 to 3.5 hours at 100°C provides optimum yield of hexosamines from plasma glycoproteins without significant destruction.

The first step in the colorimetric estimation of hexosamines is acetylation with acetyl acetone in 1.0 N sodium carbonate. In our laboratory the modified method of Boas (97) was adopted since it gave a single absorption peak (Figure 3) and a satisfactory degree of reproducibility. However, it was necessary to work out the optimal condition for acetylation since this has a very significant effect on the color yield. For this purpose a known amount of D-glucosamine standard, as well as hexosamines obtained from normal rat plasma glycoproteins, were acetylated for various periods of time, and the optical density of the product determined. The results are presented in Figure 4. The optimal acetylation time would appear to be between 40 and 60 minutes.

A standard curve for hexosamines was obtained with five different concentrations of D-glucosamine. Each value presented in Table VI and Figure 5 is the mean of five determinations for each concentration. The color intensity is shown to be proportional to the concentration of the D-glucosamine.

1. Identification of plasma glycoproteins hexosamine

Chromatographic identification of the hexosamines from human and rat plasma glycoprotein was also carried out (11). Hexosamines liberated from plasma glycoproteins and standard D-glucosamine were spotted on Whatman #1 filter paper and chromatographed for 35 hours using collodine saturated with water as the solvent system. The paper was then dried and sprayed

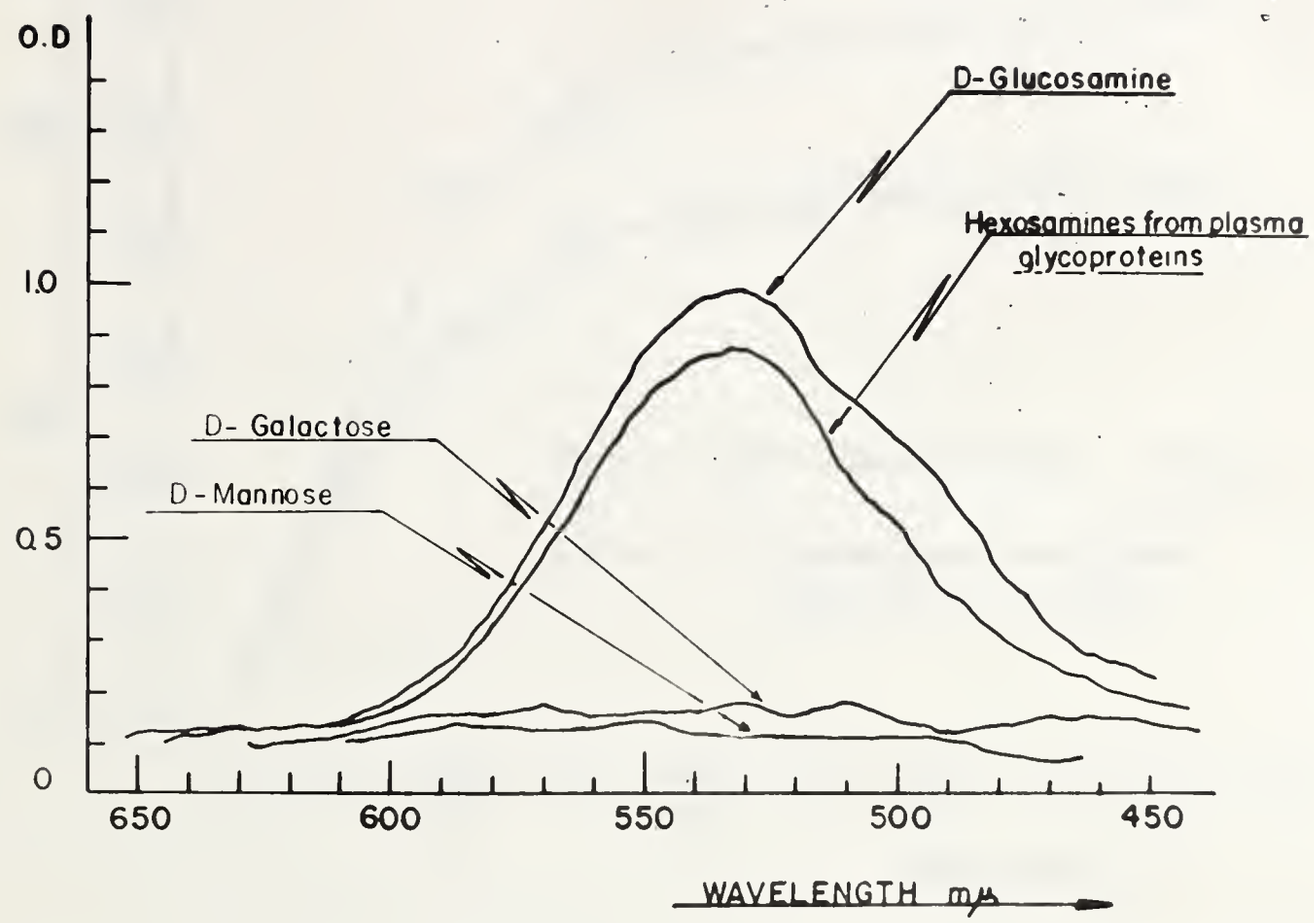


Figure 3

Absorption Curves of D-glucosamine and Hexosamines from Rat Plasma Glycoproteins

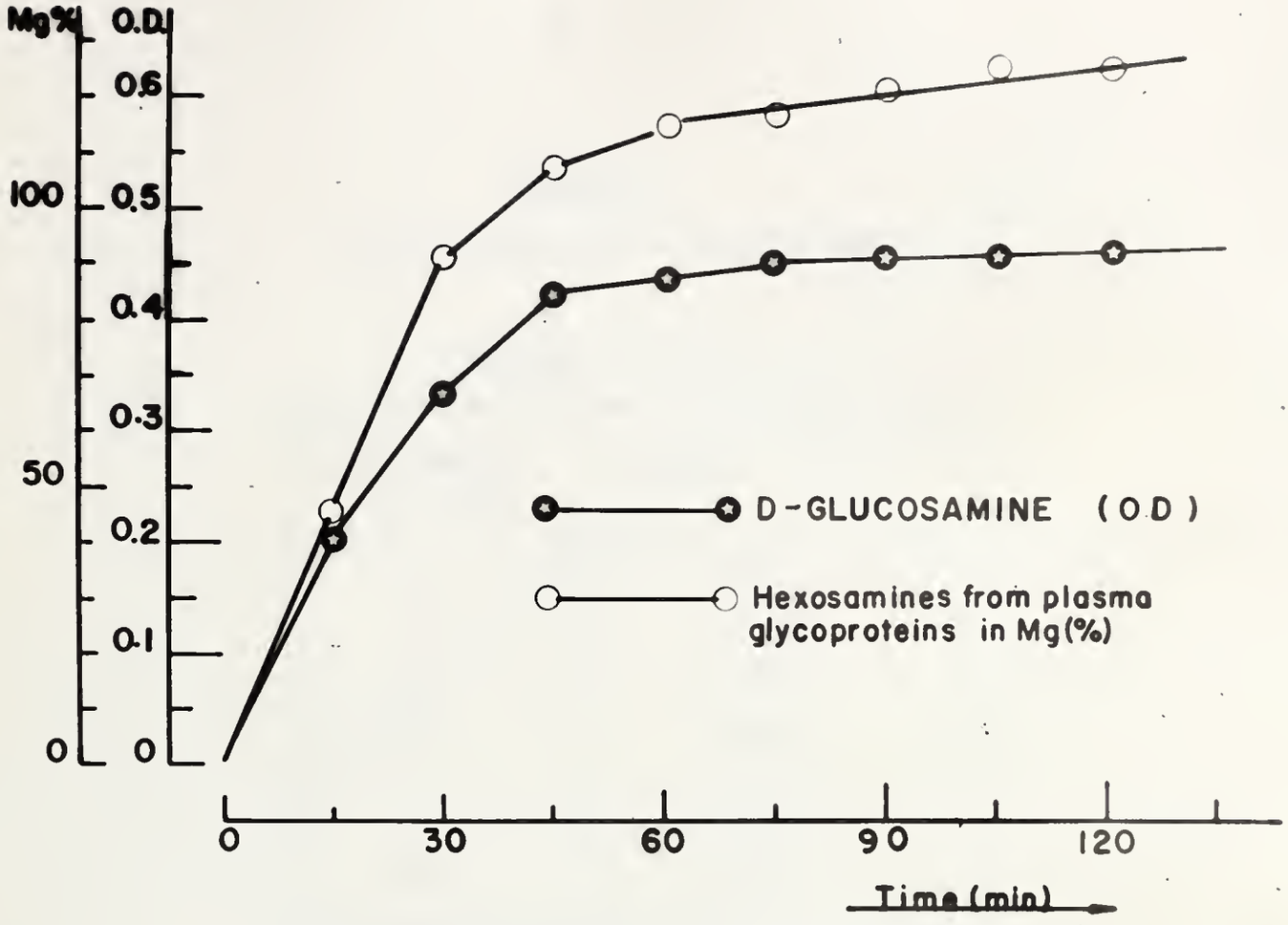


Figure 4

The Effect of Acetylation Time on D-glucosamine and Hexosamines from Rat Plasma Glycoproteins

Table VI

Standardization of D-glucosamine

Readings at 530 mμ

D-Glucosamine in μgms	Optical density
20	0.0959
40	0.1813
60	0.2609
80	0.3440
100	0.4140

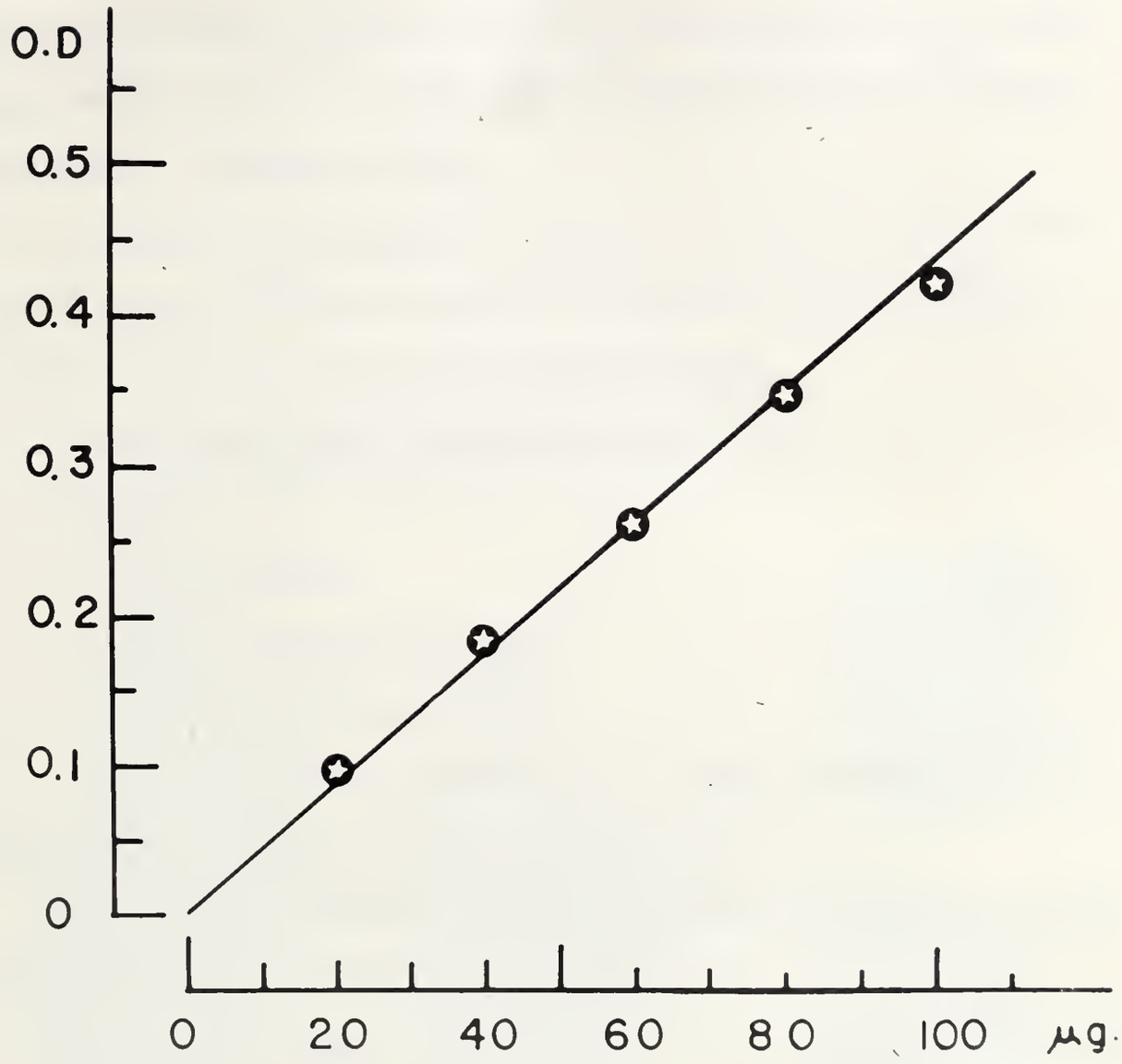


Figure 5

Relationship Between Concentration of D-glucosamine and Optical Density

with acetyl acetone reagent and heated for 10 minutes at 105°C. The dry paper was sprayed with dimethylbenzaldehyde reagent and heated for further 10 minutes at 90°C.

The paper chromatographic experiments (Figure 6) indicate that the two spots of unknown hexosamines correspond in their mobility with standard D-glucosamine and D-galactosamine.

2. Procedure used for routine analysis (97)

Reagents:

1/ 95% ethanol

2/ 3.0 N hydrochloric acid

3/ 3.0 N sodium hydroxide

4/ Acetyl acetone reagent: 1 ml of acetyl acetone in 50 ml of 1.0 N sodium carbonate, freshly prepared daily

5/ Dimethylaminobenzaldehyde dissolved in a mixture of 30 ml ethanol and 30 ml concentrated hydrochloric acid.

To 0.4 ml of plasma, 10 ml of ethanol was added, mixed and centrifuged. The protein residue was washed twice with ethanol and centrifuged. Samples were subsequently drained for 30-60 minutes. 2 ml of 3.0 N hydrochloric acid was then added and the samples were hydrolyzed for 3 to 3.5 hours at 100°C in a sand bath. They were then cooled to room temperature and the pH adjusted to 6.5-7.0 with 3.0 N sodium hydroxide and the volume made up to 20 ml with distilled water. In order to remove interfering chromogens the hydrolyzed sample was transferred into a Dowex-50 cation exchange column. The column was washed twice with 10 ml of distilled water. The hexosamines were then eluted from the resin with 8 ml of 2.0 N

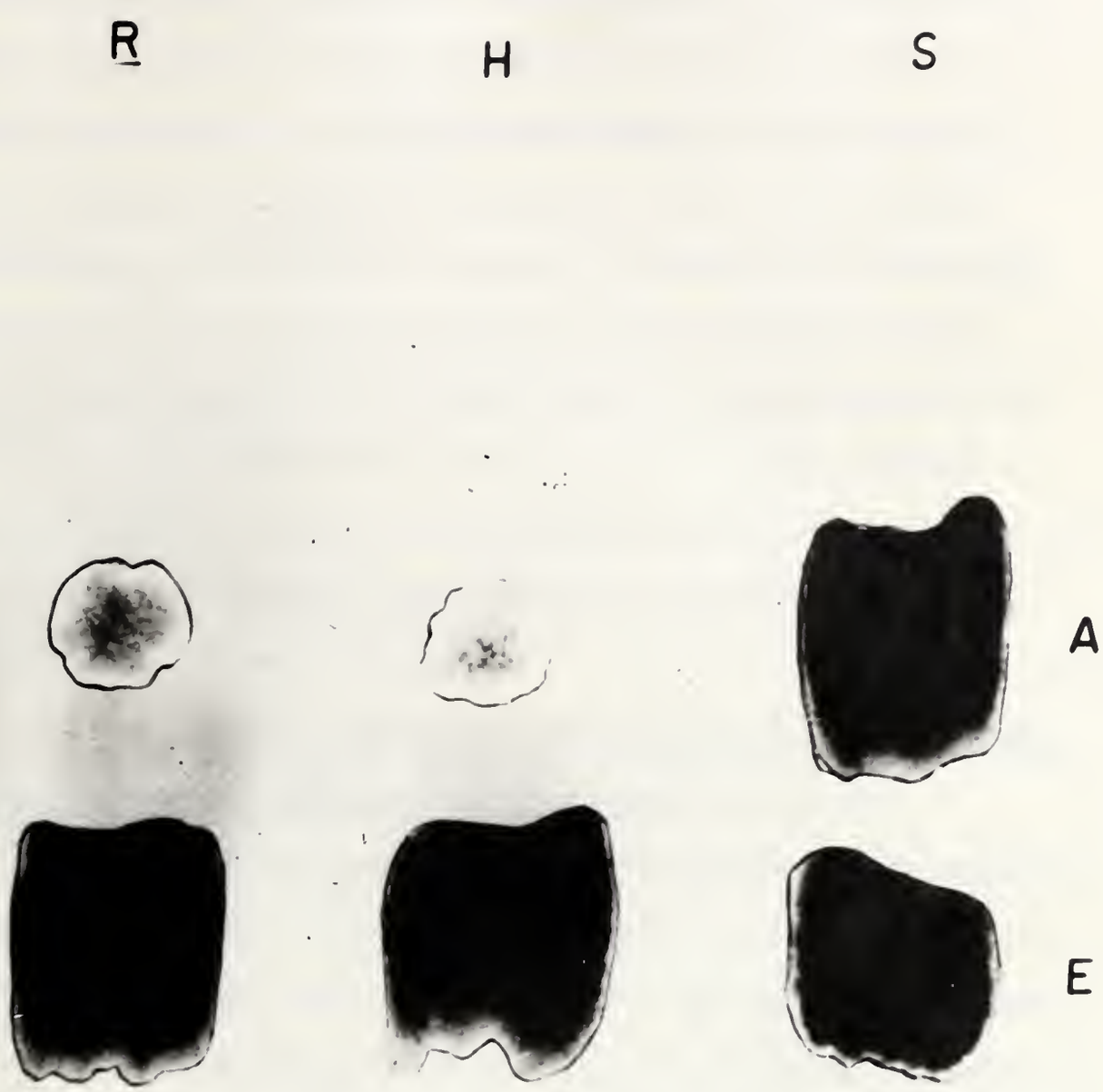


Figure 6

Paper Chromatogram of D-glucosamine, D-galactosamine and Hexosamines from Human and Rat Plasma Glycoproteins

S = Standards
H = Hexosamines from human plasma glycoproteins
R = Hexosamines from rat plasma glycoproteins
A = D-galactosamine, E = D-glucosamine

hydrochloric acid. The effluent was collected in a graduated test tube, neutralized, and its volume made up to 15 ml with water. To 1-3 ml of effluent in a stoppered centrifuge tube 1 ml of acetyl acetone reagent was added and transferred into an 85°C constant temperature water bath for 45 minutes. The samples were next cooled and 2.5 ml of 95% ethanol was added and mixed. Finally 1 ml of dimethylaminobenzaldehyde was added, mixed and the volume was made up to 10 ml with ethanol. The pink color which appeared almost instantly remained stable for many hours. Readings were taken on the Beckman DU Spectrophotometer at 530 mμ. Duplicates as well as blank were run on each specimen.

C. Isolation and quantitative estimation of N-acetylneuraminic acid from human and rat plasma glycoproteins (81, 99, 100)

There are several common color reactions used in carbohydrate chemistry. These include the color development with dimethylamino-benzaldehyde (direct Ehrlich reaction) (81), orcinol (Bial's reagent), and the resorcinol reagent of Svennerholm (99, 100) all of which give positive reactions with N-acetylneuraminic acid. However, the column chromatographic technique of Svennerholm (100) has clearly demonstrated the superiority of the resorcinol method (100) and the modified direct Ehrlich reaction (81) in this regard and for this reason these techniques have been utilized in this investigation.

The direct Ehrlich reaction (81) is the most specific, but the least sensitive, test for N-acetylneuraminic acid. The mechanism of this test is still unknown but recent evidence indicates that under mild

acid treatment with hydrochloric acid, N-acetylneuraminic acid is converted to a pyrrole-2-carboxylic acid derivative. The pyrrole compound then condenses with dimethylbenzaldehyde and gives a purple color which manifests maximum absorption at 565 mμ (Figure 7).

Bial's orcinol reaction is a time-honored test for pentose sugars, but it gives a positive reaction with N-acetylneuraminic acid as well. It was observed by Svennerholm (99) that when orcinol was replaced by resorcinol, the performance of the test was superior to that observed with the orcinol reagent. It was found that if liberated N-acetylneuraminic acid is heated at 100°C in the presence of the hydrochloric acid-resorcinol reagent, the N-acetylneuraminic acid reacts with resorcinol to give a blue color which has an absorption maximum at 580 mμ (Figure 7).

The direct Ehrlich and Svennerholm's resorcinol color reactions may be applied only to free N-acetylneuraminic acid and therefore it became necessary to establish the optimal conditions for the liberation of plasma protein-bound N-acetylneuraminic acid. The temperature and acid concentration were thoroughly studied by Svennerholm (99, 100). It was decided therefore to investigate the importance of time in relation to 0.1 N sulfuric acid hydrolysis at 80°C.

In order to establish the optimal hydrolysis time, 0.2 ml of plasma, in each of 30 test tubes, was precipitated with 95% ethanol and centrifuged. Protein residues were then washed twice with 95% ethanol and the samples were recentrifuged. The supernatant was discarded and the remaining ethanol was drained by inverting the tubes on filterpaper. To each sample

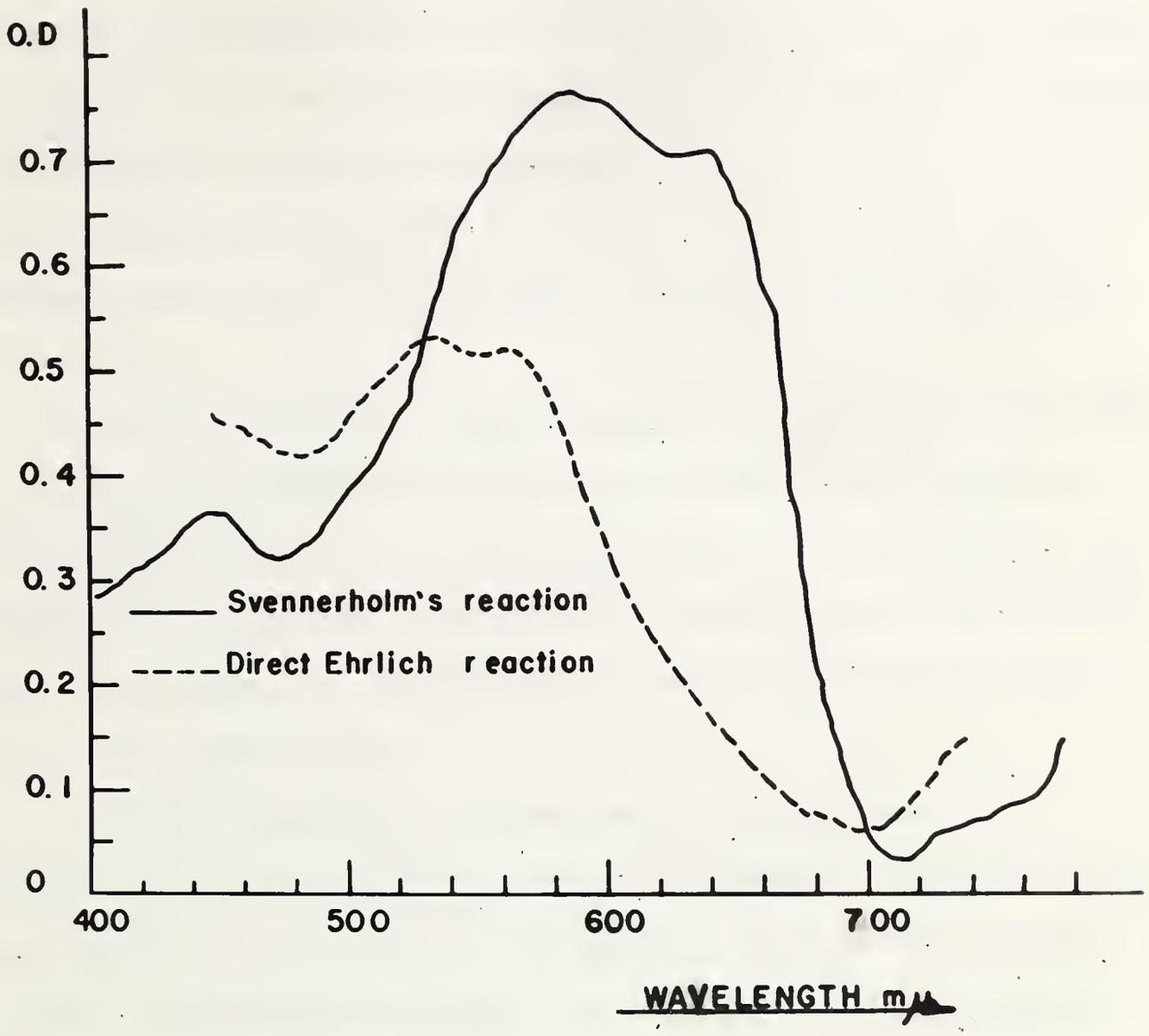


Figure 7

Absorption Curves of N-acetylneuraminic Acid Utilizing Svennerholm's Resorcinol Reaction and Direct Ehrlich Color Reaction

5 ml of 0.1 N sulfuric acid was added and hydrolyzed for various lengths of time at 80°C. The N-acetylneuraminic acid thus liberated was determined quantitatively by means of the resorcinol color reaction. The results of this experiment are presented in Figure 8. They indicate that the maximum yield occurs after 45 minutes and remains stable even after 120 minutes.

It has been shown by several researchers (21, 99, 100) that during a 60 minutes acid hydrolysis some of the protein-bound D-galactose, D-mannose and L-fucose are also released from the oligosaccharide chain. Since all the previously mentioned color reactions give positive reaction with these sugars, the validity of the results for N-acetylneuraminic acid became questionable.

There is therefore an obvious need for a simple purification method by which interfering sugars may be eliminated. Svennerholm (100) provided just such a method in his elegantly developed anion exchange column chromatographic technique. The hydrolyzate is passed through a column containing a semi-micro anion exchange resin. The N-acetylneuraminic acid is retained while the interfering sugars pass through. The N-acetylneuraminic acid is then eluted from the resin with an acetate buffer of pH 4.6 and the effluent used for the quantitative color reaction. No interference from other sugars has been observed by Svennerholm and in our laboratory. The reliability of this technique has been tested frequently in this laboratory and the recovery of standard N-acetylneuraminic acid after resin separation was 94.2-98.2%.

Svennerholm suggested 15 minutes heating time as the minimum

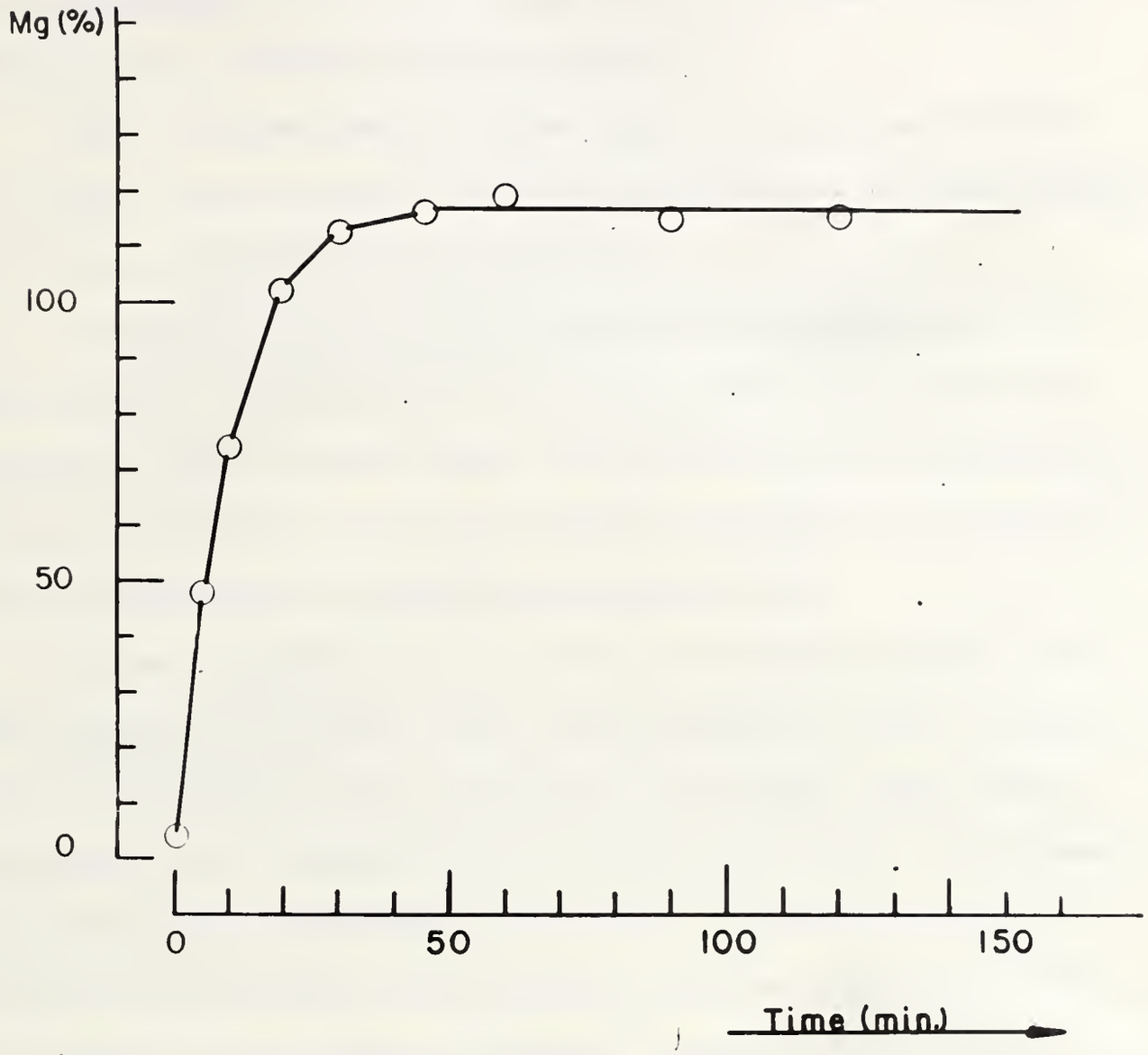


Figure 8

Relationship Between Hydrolysis Time and the Liberation of Plasma Protein-bound N-acetylneuraminic Acid

necessary for the color development at 100°C. This part of his method was reinvestigated in our laboratory using known amounts of N-acetylneuraminic acid. Results are given in Table VII.

Table VII shows that the optimal yield in color occurred after a 30 minute heating period at 100°C and therefore this has been accepted as standard procedure for our experiments.

A standard curve for N-acetylneuraminic acid was obtained with five different concentrations of N-acetylneuraminic acid. Each value presented in Table VIII and Figure 9 is the mean of five determinations for each concentration. The color intensity is shown to be proportional to the concentration of the N-acetylneuraminic acid.

Recovery experiments in connection with N-acetylneuraminic acid were carried out as follows: forty test tubes were utilized in groups of ten each. Groups A and B contained 0.2 ml of normal human plasma and groups C and D contained 0.2 ml of normal rat plasma. In all cases the proteins were precipitated with 5 ml of 95% ethanol and after 30 minutes were centrifuged. The precipitate was then washed twice with 5 ml ethanol, recentrifuged and drained. To groups B and D a known amount of N-acetylneuraminic acid standard was added as indicated in Table IX and hydrolysis, as previously described was carried out. All samples were subjected to resin purification and their N-acetylneuraminic acid content determined by the resorcinol technique. As the results in Table IX show the recovery of the added N-acetylneuraminic acid was 92.1-93.4%.

Table VII

The Relationship Between Color Development and Heating Time

Time of heating in minutes	Optical density
0	0.000
5	0.086
10	0.172
20	0.268
30	0.305
40	0.324
50	0.328
60	0.332

Table VIII

Standardization of N-acetylneuraminic Acid

Readings at 580 mμ

N-acetylneuraminic acid in μgms	Optical density
6.6	0.048
16.6	0.119
33.6	0.243
66.3	0.466
99.5	0.702

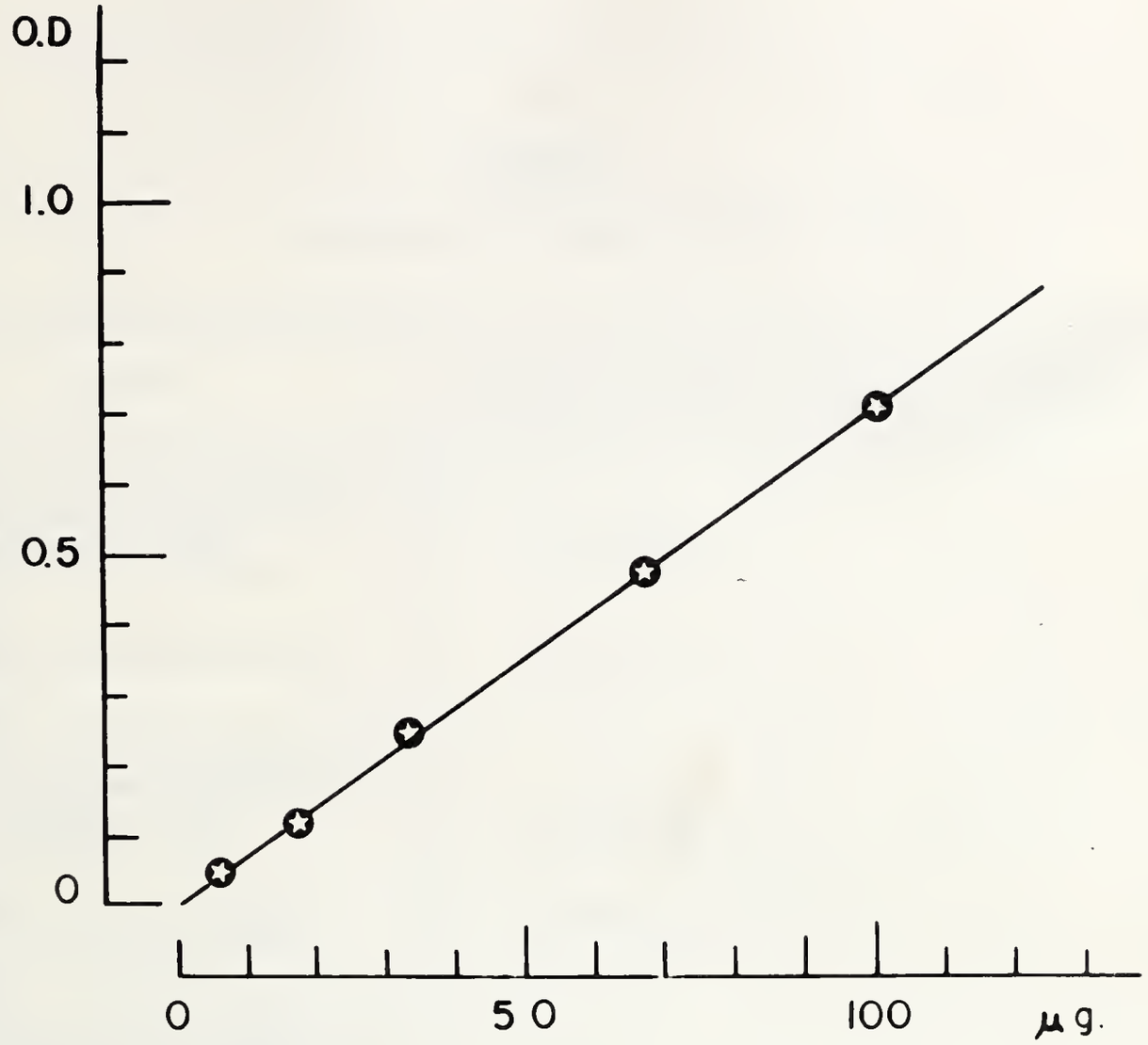


Figure 9

Relationship Between Concentration of N-acetylneuraminic Acid and Optical Density

Table IX

Recovery of N-acetylneuraminic Acid

Sample	Estimated N-acetyl-neuraminic acid	Recovery of N-acetyl-neuraminic acid in %
100 µgms of N-acetyl-neuraminic acid	100.00 ±1.8	
A. 0.2 ml of human plasma	125.08 ±2.4	
B. 0.2 ml of human plasma and 50 µgms of N-acetyl-neuraminic acid	171.80 ±3.1	93.4
C. 0.2 ml of rat plasma	167.12 ±2.5	
D. 0.2 ml of rat plasma and 50 µgms of N-acetyl-neuraminic acid	213.20 ±3.3	92.1

1. Identification of type of N-acetylneuraminic acid

The actual identification of the type of N-acetylneuraminic acid which is present in the plasma protein required further purification, crystallization, and finally paper chromatographic analysis (101).

To 100 ml of either human or rat plasma 400 ml of 95% ethanol was added, mixed and heated in a water bath at 80°C for 40 minutes. This precipitated the plasma proteins and after cooling and centrifuging the residue was washed twice in 400 ml of ethanol and centrifuged. Subsequently it was mixed with 1000 ml of 0.1 N sulfuric acid and left for 24 hours at 2°C. At the end of this time the suspension was centrifuged and the acid supernatant was discarded. The precipitate was hydrolyzed with 200 ml of 0.1 N sulfuric acid at 80°C for 1 hour in order to liberate the protein-bound N-acetylneuraminic acid. The hydrolyzate was cooled, centrifuged and the supernatant was removed. This hydrolysis was repeated two more times and the supernatants were combined. The pH of the combined supernatant was adjusted with saturated barium hydroxide to 6.0 and the precipitate of barium sulfate was filtered off. The filtrate was passed through a Dowex-50 cation exchange column, and then a Dowex-1 anion exchange column. When all the filtrate had gone through the anion exchange resin the column was washed with 1000 ml of distilled water in order to remove the interfering sugars, after which the N-acetylneuraminic acid was eluted with 0.3 N formic acid. The effluent was concentrated *in vacuo* to one-fifth its original volume and lyopholized to dryness.

The lyopholized sample was dissolved in 2 ml of distilled water.

Twenty ml of methyl alcohol were added and the mixture warmed to 40°C. Then 30 ml of anhydrous diethyl ether was added. Some precipitation was observed which was removed by means of filtration. To the effluent another 20 ml of ether was added. After mixing, immediate crystallization of the N-acetylneuraminic acid could be observed. The crystals were collected after 48 hours and used for paper chromatographic analysis.

For identification of N-acetylneuraminic acid the unknown sample and N-acetylneuraminic acid standard were spotted on Whatman #1 paper using n-butanol-n-propanol-0.1 N hydrochloric acid (1:2:1) as the solvent system. After 40 hours the chromatographic paper was dried and sprayed with resorcinol-butanol reagent and dried at 100°C for 15 minutes.

The chromatograms indicate that normal human and rat plasma contain one type of sialic acid, namely N-acetylneuraminic acid.

2. Procedure used for routine analysis (81, 99, 100)

Reagents and preparation of Dowex-1 anion exchange resin:

1/ 95% ethanol

2/ 0.1 N sulfuric acid

3/ 2.0 N sodium hydroxide

4/ 2.0 N hydrochloric acid

5/ 2.0 N sodium acetate

6/ 0.1 N acetic acid

7/ 1.0 M pH 4.6 acetic acid-sodium acetate buffer

8/ Anion exchange resin (Dowex-1 200-400 mesh resin)

9/ Standard N-acetylneuraminic acid

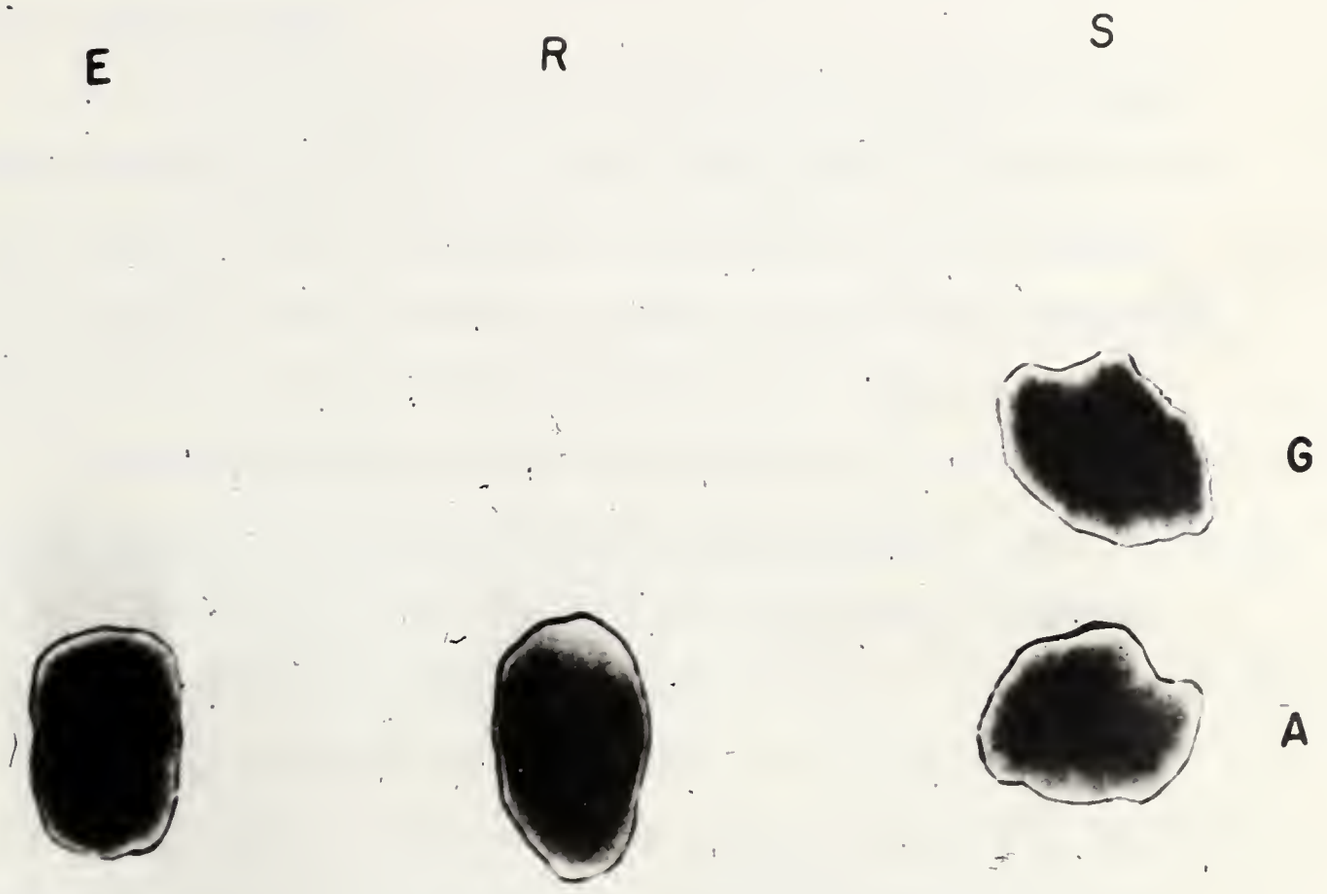


Figure 10

Paper Chromatogram of N-acetylneuraminic Acid, N-glycolylneuraminic Acid and N-acetylneuraminic Acid from Human and Rat Plasma Glycoproteins

S = Standard
R = N-acetylneuraminic acid from rat plasma glycoproteins
E = N-acetylneuraminic acid from human plasma glycoproteins
G = N-glycolylneuraminic acid
A = N-acetylneuraminic acid

10/ 0.1 M cupper sulfate

11/ Isoamyl alcohol

12/ Resorcinol reagent: 0.2 gm of resorcinol dissolved in 10 ml of distilled water and 80 ml of concentrated hydrochloric acid and 0.25 ml of 0.1 M cupper sulfate added. The volume is made up to 100 ml with distilled water.

The resin was converted to the chloride form with 2.0 N hydrochloric acid at 100°C for 30 minutes. This process was repeated once more. Then the resin was converted to the acetate form by passing 2.0 N ammonium acetate through the column. The resin was washed with water and then suspended in one volume of 0.1 N acetic acid.

The working column was made as follows: 10 ml of the resin-acetic acid mixture was pipetted into a chromatographic tube (dimensions 1 x 25 cm) and washed with 10 ml of 0.1 N acetic acid. The actual dimension of the resin in the column is 1 x 6 cm.

The column can be regenerated after the elution is completed, with 10 ml solutions of each of in the following order given: 2.0 N sodium hydroxide, distilled water, 2.0 N sodium acetate and 0.1 N acetic acid.

To 0.2 ml of plasma 5 ml of ethanol was added, mixed and centrifuged. The protein residue was washed twice with ethanol and centrifuged. The samples were drained for 30-60 minutes, then 5 ml of 0.1 N sulfuric acid was added and hydrolysis carried out for 1 hour at 80°C. The hydrolyzate was then cooled to room temperature and transferred into the Dowex-1 anion exchange column. The column was washed twice with 5 ml of distilled water.

Then the N-acetylneuraminic acid was eluted from the resin with 9 ml of acetate buffer (pH 4.6). The effluent was collected in a graduated test tube and its volume made up to 10 ml with water.

The N-acetylneuraminic acid content of the effluent was estimated by the Svennerholm (99) resorcinol method.

Resorcinol method: To 2 ml of the effluent in a centrifuge tube 2 ml of the resorcinol reagent was added and mixed. The stoppered tubes were heated for 30 minutes in a boiling water bath and then after cooling to room temperature, 5 ml of isoamyl alcohol was pipetted into the tubes which were shaken vigorously and chilled in an ice bath for 10 minutes. The samples were then centrifuged for 3-5 minutes and the colored isoamyl alcohol layer was removed and read at 580 mμ on a Beckman DU Spectrophotometer. The standard N-acetyl-neuraminic acid and blank were treated under the same conditions.

D. Isolation and quantitative estimation of L-fucose from human and rat plasma glycoproteins

Estimations of L-fucose in human and rat plasma glycoprotein have been carried out by the method of Dische and Shettles (12, 102, 103) using the sulfuric acid-cysteine reaction which involves two separate steps:

a/ the cleavage of the glycosidic linkage by which the monosaccharide is attached to the oligosaccharide molecule and its subsequent dehydration to 5-methylfurfural (103, 104),

b/ condensation of 5-methylfurfural with cysteine reagent giving a light yellow color with maximum absorption at 396 mμ (Figure 11 and 12).

It was observed (12) that this sugar compound is a minor constituent of plasma glycoproteins. During acid hydrolysis the other carbohydrate constituents, such as galactose and mannose, are released simultaneously with L-fucose and consequently give a color reaction with cysteine reagent. To eliminate the interference of other sugars, an appropriate hydrolysis time must be established. For this purpose pure L-fucose, D-glucose, D-galactose, D-mannose, D-glucosamine, N-acetylneuraminic acid and plasma glycoproteins were dehydrated for various lengths of time at 100°C with sulfuric acid. The samples were then subjected to the condensation reaction with cysteine. Each sample was scanned on a Bousch and Lomb 505 spectrophotometer between 340 and 500 mμ and the results are shown in Figures 11 and 12.

Figure 11 summarizes the absorption curves of various carbohydrates after 3 minutes dehydration. It is apparent that each hexose reacts with cysteine and gives a distinct absorption maximum very close to that of the L-fucose. Since D-galactose and D-mannose are constituents of the plasma glycoproteins their interference under these circumstances is considerable. On the other hand the interference of D-glucosamine and N-acetylneuraminic acid is negligible.

Results in Figure 12 were obtained when the heating period was increased from 3 to 10 minutes. The spectrophotometric analysis clearly demonstrates that D-glucose, D-galactose and D-mannose exhibit a weak maximum at 410 mμ compared to the very sharp peaks obtained after 3 minutes hydrolysis. On the other hand the absorption spectra of the pure L-fucose and the L-fucose of plasma glycoproteins are almost

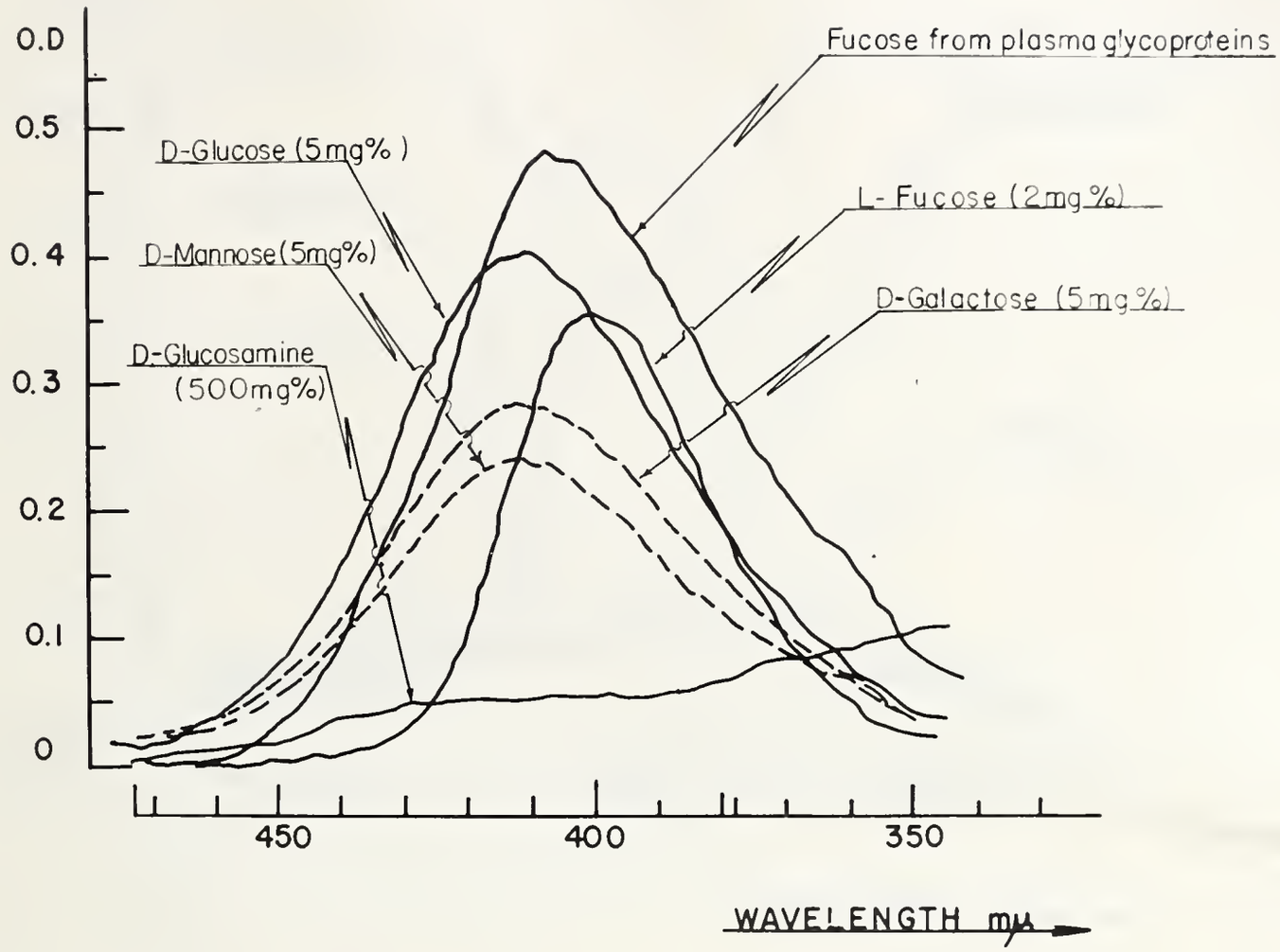


Figure 11

Absorption Curves of L-fucose, D-glucose, D-galactose, D-mannose, D-glucosamine and Fucose from Human and Rat Plasma Glycoproteins After 3 minutes Heating Time

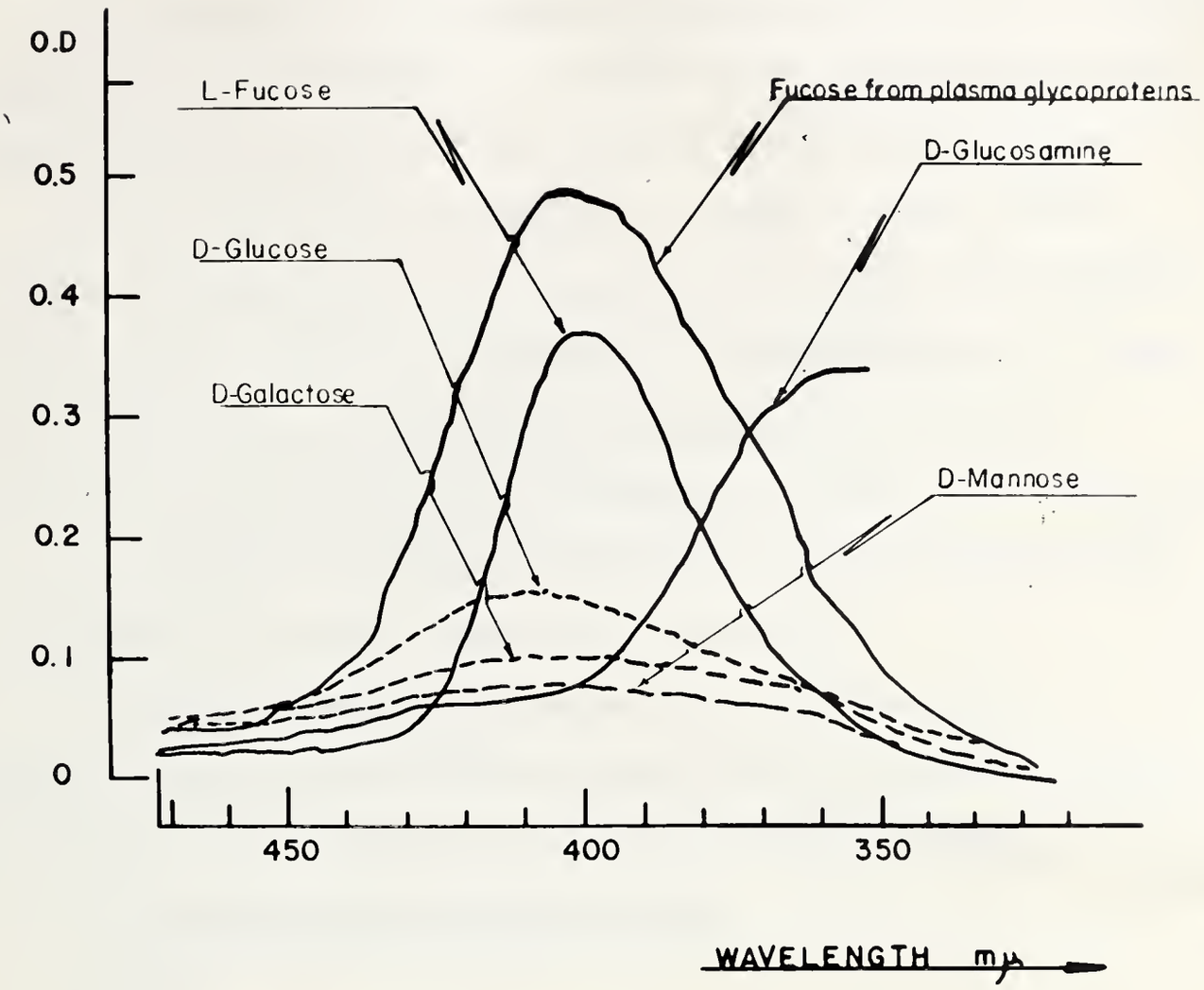


Figure 12

Absorption Curves of L-fucose, D-glucose, D-galactose, D-mannose, D-glucosamine and Fucose from Human and Rat Plasma Glycoproteins After 10 minutes Heating Time

identical at both hydrolysis times.

Hence the sulfuric acid-cysteine reaction, modified by an increased dehydration period, enables one to estimate fucose quantitatively from various biological material in the presence of other carbohydrates.

For the quantitative estimation of L-fucose in plasma glycoproteins a calibration curve was obtained. Pure L-fucose from Pfanstiehl Chemical Company was used as a standard in a concentration of 5-25 µgms per ml. Each point in the standard curve represents at least five replicates. Experimental results are tabulated in Table X and Figure 13.

The values of the standard thus obtained are proportional to the concentration in the range between 5-25 µgms per ml.

A recovery experiment was carried out with a known amount of L-fucose added to the precipitated plasma protein. One group of samples was hydrolyzed for 3 minutes, the second group for 10 minutes.

1. Procedure used for routine analysis

Reagents:

1/ L-fucose standard - 5, 10, 15, 20 and 25 µgms per ml

2/ 95% ethanol

3/ Cysteine-hydrochloride, 3% solution in water

4/ Sulfuric acid-water mixture (6:1) v/v

Duplicates as well as a sample blank were run on each specimen.

To each test tube were added 0.1 ml of plasma and 5 ml ethanol with mixing. Samples were centrifuged in a Serval centrifuge at 2000xg (4000 RPM) for 10-15 minutes at 5°C, the ethanol was decanted and the precipitate was washed with 5 ml ethanol. The residue was dissolved in

Table X

Optical Density of L-fucose Standards

Concentration of L-fucose in µgms per ml	Optical density
5	0.110
10	0.202
15	0.313
20	0.399
25	0.536

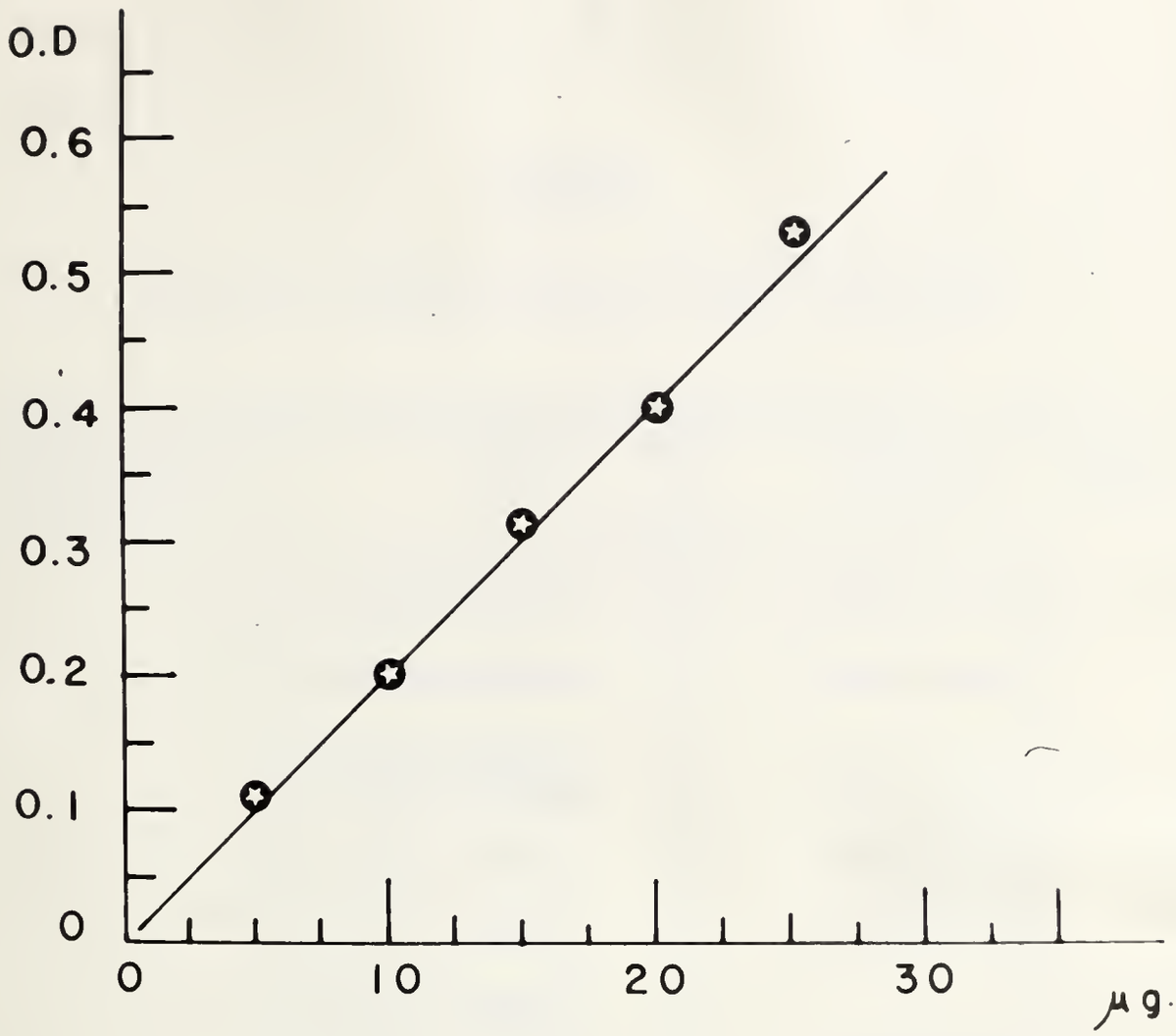


Figure 13

Relationship Between Concentration of L-fucose and Optical Density

Table XI

Recovery of L-fucose Added to Plasma Glycoprotein

Hydrolysis time in minutes	Sample	L-Fucose in µgms	Recovery in %
3	10 µgms L-fucose	10.00 ±0.25	
	0.1 ml of plasma	9.60 ±0.39	
	0.1 ml of plasma + 10 µgms L-fucose	19.04 ±0.34	94.4
10	10 µgms L-fucose	10.00 ±0.20	
	0.1 ml of plasma	10.16 ±0.36	
	0.1 ml of plasma + 10 µgms L-fucose	19.70 ±0.35	95.4

Results are expressed in µgms, ± standard deviation

1 ml of 0.2 N sodium hydroxide and transferred to an ice bath and 4.5 ml of sulfuric acid-water mixture was added and mixed. The tubes were then immersed in a boiling water bath for 10 minutes, after which they were cooled in tap water. To each tube 0.1 ml of cysteine reagent was added (except to the plasma blank sample in order to correct for non-specific color development) with instant mixing. The greenish-yellow color which appeared almost instantly remained stable for many hours. Readings were taken on the Beckman DU Spectrophotometer at 396 and 430 mμ, with distilled water used to set the instrument to zero.

The fucose content of the plasma glycoprotein was calculated from the dichromatic reading obtained at 396 and 430 mμ.

V. RESULTS

A. Human plasma glycoproteins in various disease states including carcinoma

Table XII summarizes our findings with respect to plasma galactose-mannose levels in the entire series of 648 patients. A significant increase in plasma galactose-mannose is apparent in patients suffering from malignant disease as compared both with normal controls and patients with non-malignant disease. Patients suffering from non-malignant disease demonstrate a significant elevation over normal controls although of lesser magnitude.

The distribution curves of the plasma galactose-mannose levels in normal, non-malignant and malignant disease groups are illustrated in Figures 14 and 15. In Figure 15 values have been expressed as a percentage of each sub group, since the differences in size of the sub groups make comparative interpretation difficult from Figure 14. The plasma galactose and mannose levels in malignant disease (excluding carcinoma of the breast) and carcinoma of the breast are depicted separately in Figure 16. The distribution curves show clearly that the magnitude of the increase is significantly different in those groups of malignancy.

A review of the plasma protein-bound hexose (galactose-mannose) levels in the group of patients suffering from non-malignant disease suggests some interesting trends, as indicated in Table XIII. It should be noted again that these patients represented surgical admissions to a general hospital and as such are not indicative of the total spectrum of

Table XII

Plasma Galactose-Mannose Levels in Various Human Diseases

Diagnosis	Number of cases	Galactose-mannose
Normal	101	81.7 ±0.83
Non-malignant disease	386	112.9 ±1.59*
Malignant disease	161	140.5 ±2.33*

Results expressed as mg per 100 ml plasma, ± standard error

* = P less than 0.01

$$\text{Standard error} = \frac{\text{Standard deviation}}{\sqrt{\text{Number of samples}}}$$

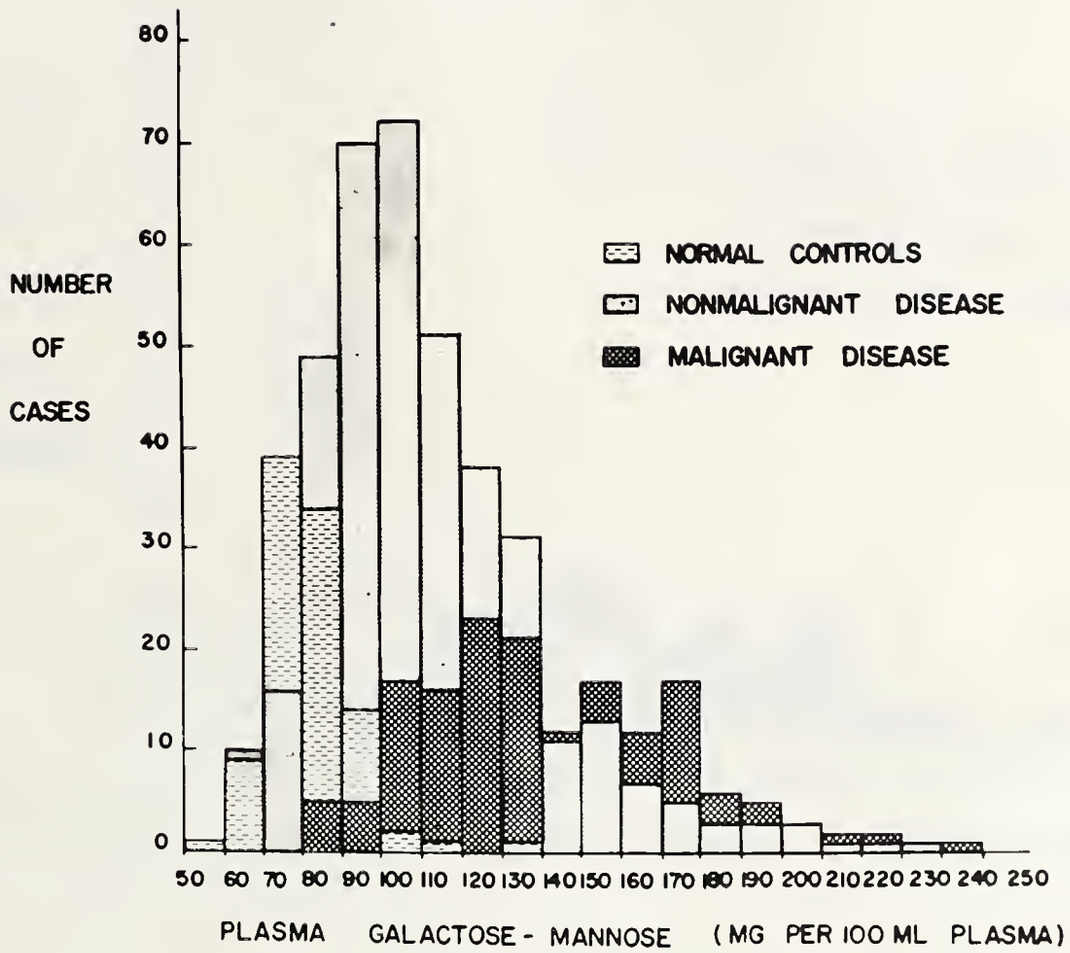


Figure 14

Plasma Galactose-Mannose Levels in Various Human Disease States

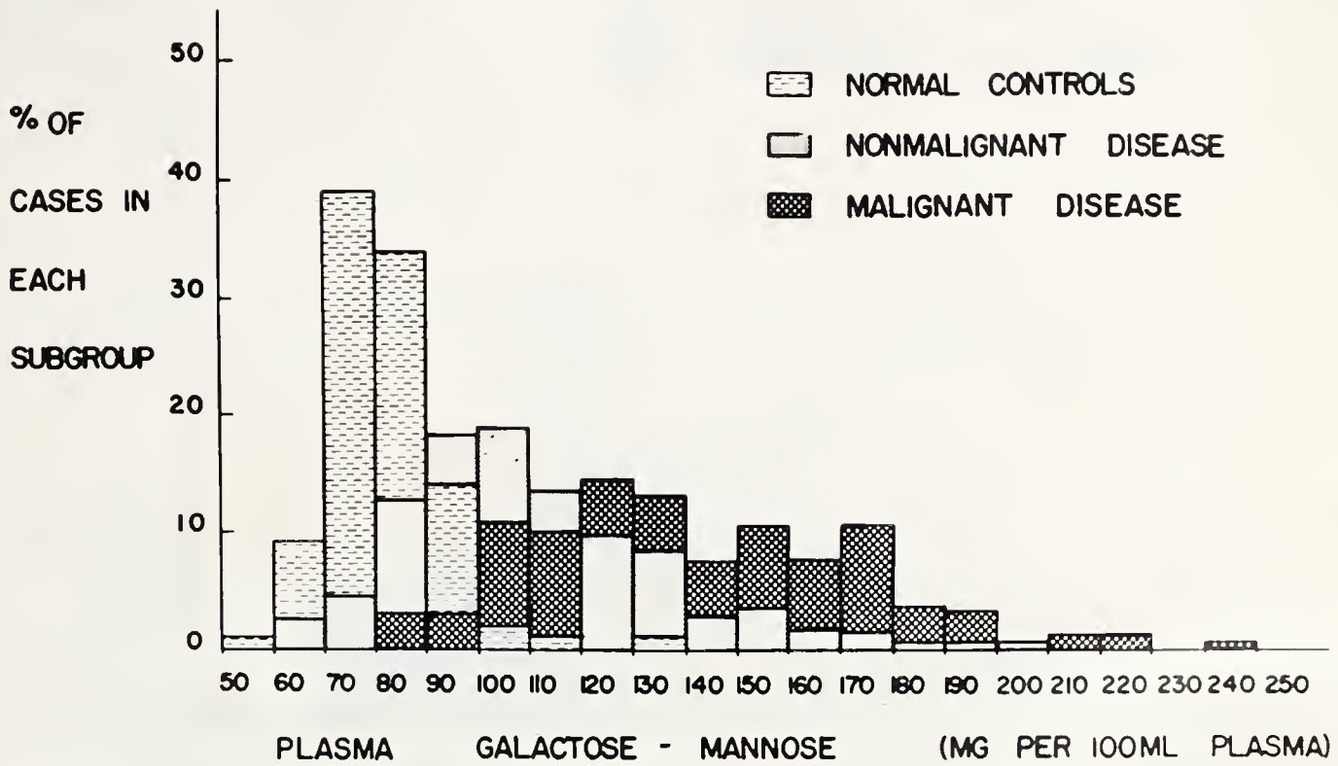


Figure 15

Plasma Galactose-Mannose Levels in Various Human Disease States Expressed as a Percentage of Each of the Three Sub Groups

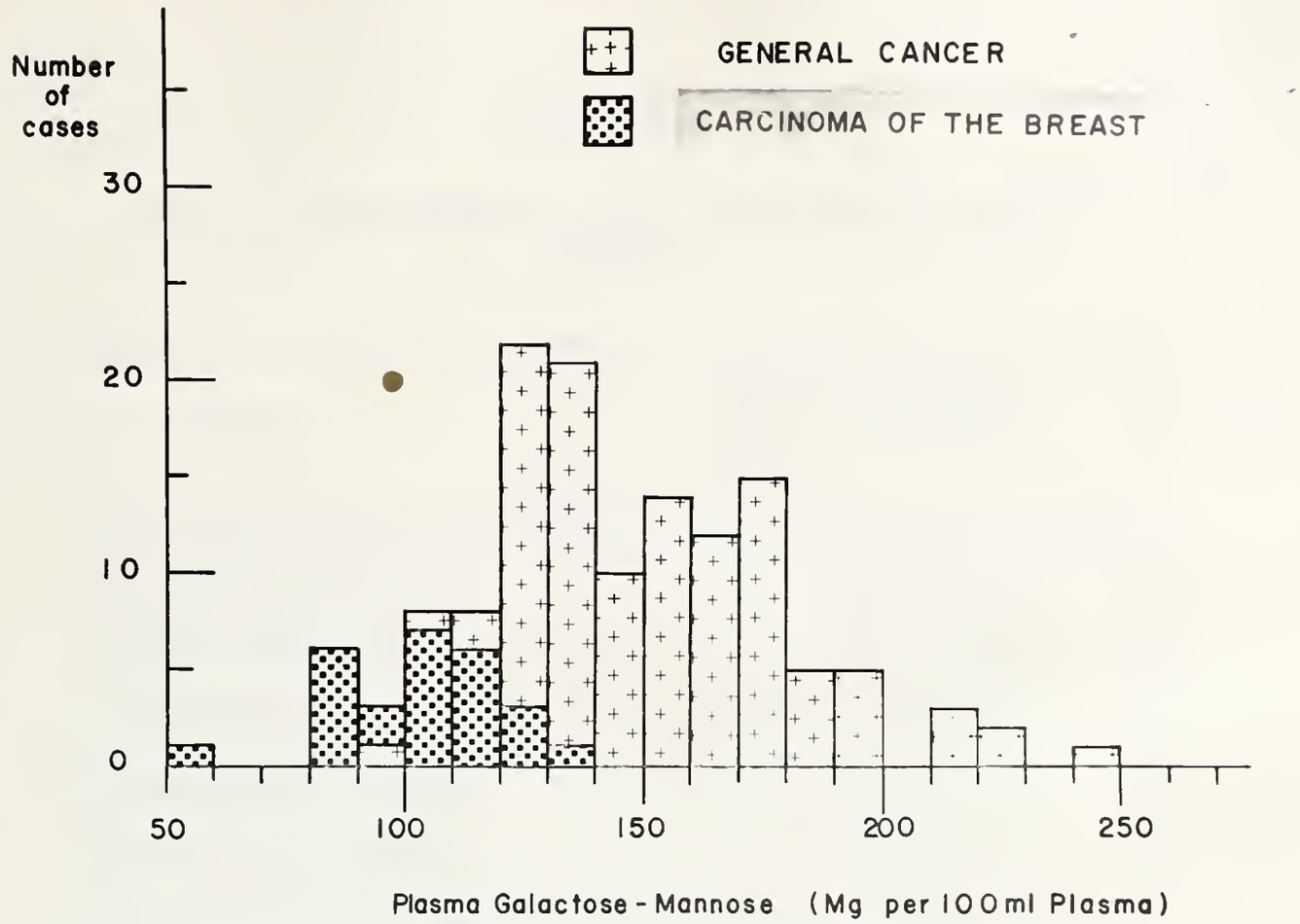


Figure 16

Plasma Galactose-Mannose Levels in Malignant Disease (Excluding Carcinoma of the Breast) and Carcinoma of the Breast

Table XIII

Plasma Galactose-Mannose Levels in Human Non-Malignant Disease

Diagnosis	Number of cases	Galactose-mannose
Cardiovascular disease	49	115.6 ±1.4
Peptic ulceration	16	125.9 ±6.1
Diabetes mellitus	11	128.0 ±6.1
Urinary tract disease	27	133.7 ±9.7
Miscellaneous disease	283	109.1 ±1.6

Results expressed as mg per 100 ml plasma, ± standard error

non-malignant disease. There were, for example, no cases of tuberculosis, active rheumatoid arthritis, or infectious hepatitis in this group. It is to be noted, however, that even with this provision four broad groups of diseases tended to have average plasma levels of protein-bound hexose in excess of those encountered in the remaining group of patients with non-malignant disease. This was particularly true of patients with disease of the urinary tract, patients with diabetes mellitus admitted to the surgical service usually because of complicating vascular disease, and patients with peptic ulceration.

Table XIV records the interesting observation that the plasma of patients with localized carcinoma of the breast gave protein-bound galactose-mannose levels within the normal range, a finding at variance with that in all other types of malignant disease investigated and at variance with those cases of carcinoma of the breast with proved metastases. Similar findings were obtained by Almquist and Lausing (22) and Shetlar et al (36).

Table XV summarizes our findings in the plasma of a group of 112 patients in which all six carbohydrate constituents of plasma glycoproteins were determined. In the non-malignant disease group the only significant variation from the normal pattern was the hexosamines (glucosamine and galactosamine) which attained a level comparable to that observed in patients with malignant disease. In clinically localized carcinoma of the breast the levels of five individual carbohydrates of plasma glycoproteins were not significantly different from those observed in non-malignant disease. However, the plasma fucose content

Table XIV

Plasma Galactose-Mannose Levels in Human Malignant Disease

Diagnosis	Number of cases	Galactose-mannose
Malignant disease (excluding breast)	129	147.3 ±2.21*
Carcinoma of the breast	27	102.2 ±3.63
Carcinoma of the breast with metastases	5	172.1 ±7.30*

Results expressed as mg per 100 ml plasma, ± standard error

* = P less than 0.01

Table XV

Galactose, Mannose, Glucosamine, Galactosamine, N-acetylneuraminic Acid and Fucose Levels in Human Diseases

Diagnosis	Number of cases	Hexose (galactose-mannose) #	Hexosamine (glucosamine-galactosamine) #	N-acetyl-neuraminic acid #	Fucose #	Total carbo-hydrate #
Normal	41	81.7 ±0.8	68.7 ±1.2	58.3 ±1.3	8.2 ±0.6	216.9
Malignant disease excluding breast	21	147.7 ±3.2*	148.2 ±6.6*	105.3 ±4.6*	12.6 ±0.7*	413.8
Carcinoma of breast	15	109.1 ±3.2	149.5 ±11.2*	89.9 ±3.7	12.0 ±0.5*	360.5
Carcinoma of breast with metastases	4	166.1 ±5.8*	202.8 ±5.8*	104.4 ±5.4*	14.4 ±0.5*	487.7
Non-malignant disease	31	97.8 ±2.4	143.3 ±5.2*	74.6 ±3.4	8.6 ±0.5	324.3

= Results expressed as mg per 100 ml plasma, ± standard error

* = P less than 0.01

was significantly elevated as compared with the normals and with patients suffering from non-malignant disease. The fucose levels in clinically localized carcinoma of the breast are, in fact, comparable to those observed in patients with all other types of malignant disease investigated. In patients with malignant disease, excluding the breast, and those with breast carcinoma with proven metastases all six carbohydrate constituents of glycoproteins were markedly elevated over the control values.

In ten patients with malignant disease it has been possible to follow, by means of serial determination of plasma glycoproteins, the response to treatment.

Case #1: The first patient (UAH #190953) was proven at laparotomy to have extensive inoperable adenocarcinoma involving the left hemipelvis, the primary origin of which could not be determined. The plasma protein-bound galactose-mannose levels during hospital treatment are recorded in Figure 17. It is to be noted that the plasma galactose and mannose level rose in spite of intensive X-ray therapy and that she succumbed to her disease.

Case #2: The same was true of the second patient (UAH #187922). This adult male had a lympho-epithelioma of the nasopharynx with generalized metastases. The diagnosis in this patient was first suspected at laminectomy for spinal cord compression due to tumour.

As shown in Figure 18, treatment with X-ray and various chemo-therapeutic agents failed to return his elevated plasma galactose-mannose content to the normal range. His clinical course was one of

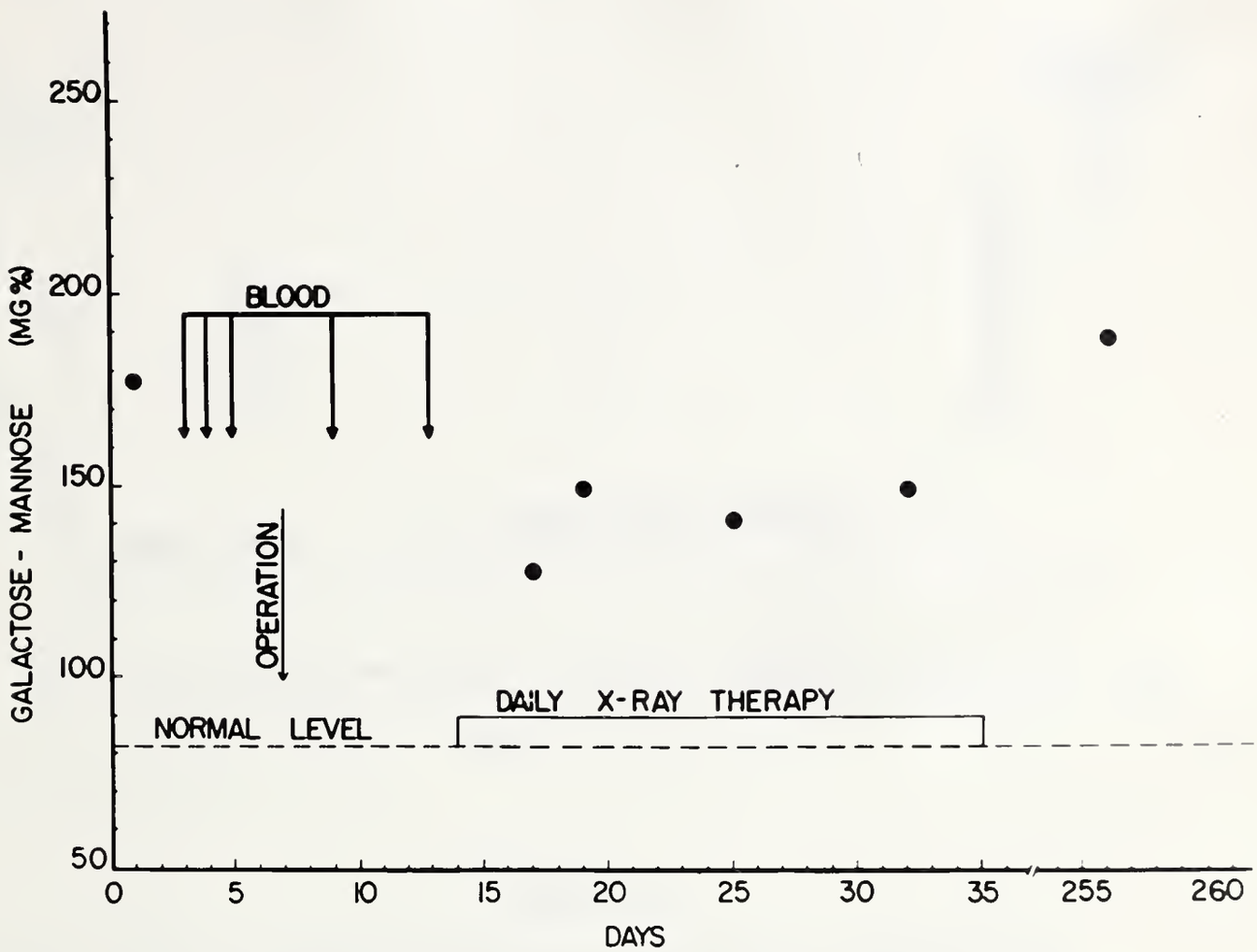


Figure 17

Serial Plasma Galactose-Mannose Levels in Patient Suffering from Widespread Adenocarcinoma Unresponsive to Treatment

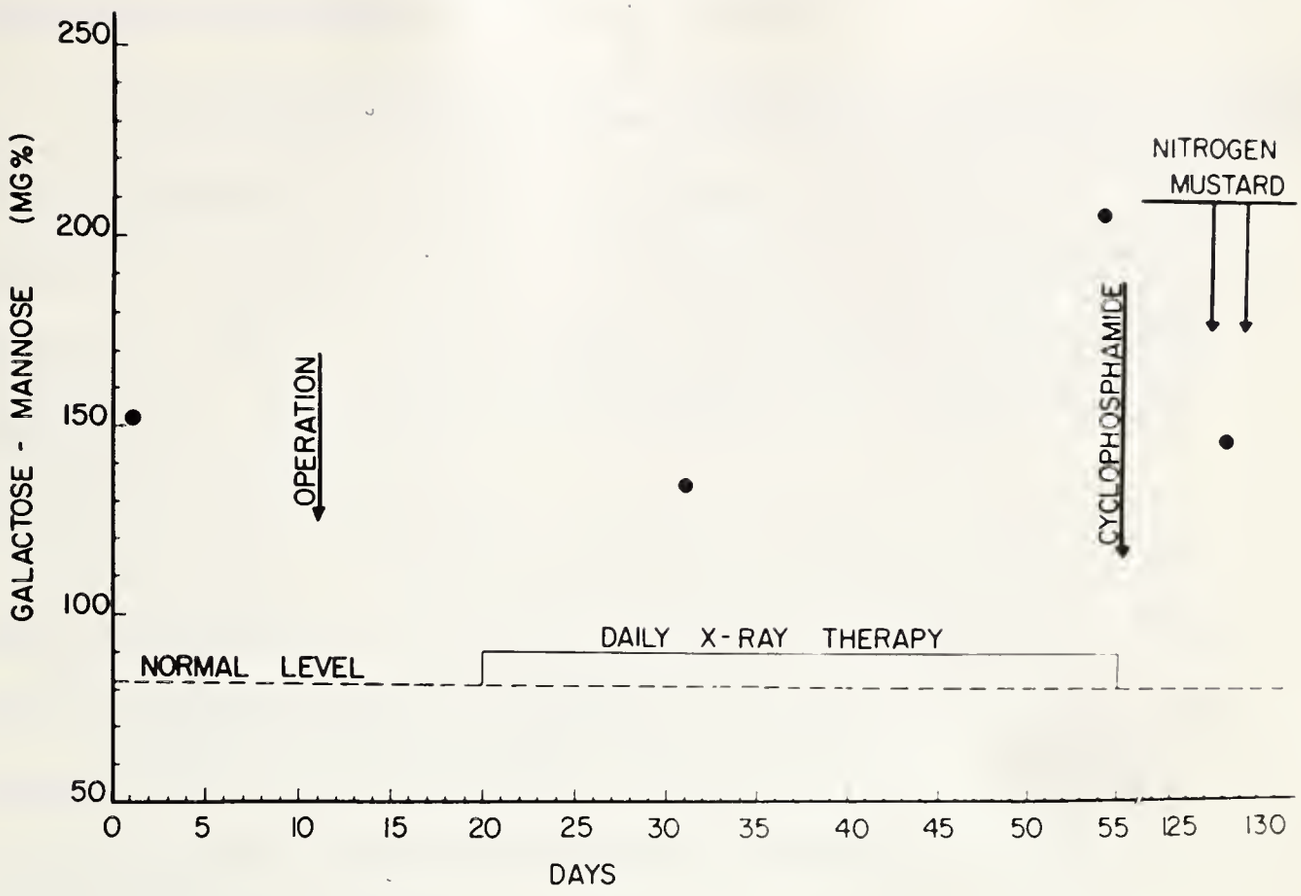


Figure 18

Serial Plasma Galactose-Mannose Levels in Patient Suffering from Metastatic Lymphoendothelioma Unresponsive to Treatment

progressive deterioration terminating in death.

Case #3: This particular patient (UAH #99172) was admitted June 11th, 1962 and underwent an exploratory laparotomy on June 19th. The omentum contained a carcinomatous mass which originated in the stomach and also invaded the liver. No curative surgery was attempted. Biopsy revealed adenocarcinoma of the stomach with metastases. The patient died on July 5th of the same year.

The results in Table XVI show that the initially high hexose (galactose-mannose) and hexosamine (glucosamine-galactosamine) levels remained markedly elevated throughout the period of observation. Although a marked increase in plasma fucose was observed, the plasma N-acetylneuraminic acid did not show any significant alteration during this period of time.

Table XVI

Serial Plasma Glycoprotein Levels in a Patient with Metastatic Adenocarcinoma of the Stomach

Date	Galactose-mannose	Fucose	Hexosamine	N-acetylneuraminic acid
June 20	162.9	10.1	151.5	80.0
June 27	158.0	12.2	145.6	91.4
July 4	151.5	17.7	184.2	80.0

Results expressed as mgm per 100 ml plasma

Case #4: This patient (UAH #41695) was admitted on the June 27th, 1962 and underwent an exploratory laparotomy on July 5th. The operation confirmed the presence of a large nodular undifferentiated carcinoma of pancreas with metastases. No curative surgery was attempted.

Pre-operative estimation of the protein-bound carbohydrates demonstrated that fucose and hexosamine were significantly elevated. Four days post-operatively a further elevation in all levels was manifest except in the case of fucose which showed a decreased plasma level.

Table XVII

Serial Plasma Glycoprotein Levels in a Patient with Carcinoma of the Pancreas

Date	Galactose-mannose	Fucose	Hexosamine	N-acetylneuraminic acid
July 3	116.5	18.35	143.1	74.4
July 9	172.6	13.40	176.0	100.2

Results expressed as mgm per 100 ml plasma

Case #5: The patient (UAH #217746) was admitted on June 27th, 1962 and underwent a laparotomy on July 16th, which revealed a large inoperable gastric carcinoma.

All carbohydrate constituents of the plasma glycoprotein were elevated pre-operatively. Further increase in the plasma fucose and hexosamines are recorded 2 and 9 days post-operatively.

Table XVIII

Serial Plasma Glycoprotein Levels in a Patient with Inoperable Gastric Carcinoma

Date	Galactose-mannose	Fucose	Hexosamine	N-acetylneuraminic acid
July 11	153.2	11.18	154.0	95.4
July 18	157.5	13.81	142.0	96.2
July 25	148.9	14.60	174.5	92.9

Results are expressed as mgm per 100 ml plasma

Case #6: This patient (UAH #213270) had previously been in hospital in February 1962 at which time an abdomino-perineal resection had been performed for adenocarcinoma of the rectum. Following this procedure a perineal sinus developed which on biopsy proved to be the result of recurrent carcinoma. He was therefore readmitted to hospital on July 2nd for excision of his perineal recurrence on July 9th. The biochemical studies reported were carried out at the time of this latter admission.

Results in Table XIX show that this patient had an initially normal plasma glycoproteins level. However, a dramatic rise in all carbohydrate constituents of plasma glycoproteins was demonstrated which coincided with the patient's general deterioration which terminated in death.

Table XIX

Serial Plasma Glycoprotein Levels in a Patient with Recurrent Carcinoma

Date	Galactose-mannose	Fucose	Hexosamine	N-acetylneuraminic acid
July 24	87.7	11.48	131.0	59.7
July 31	177.8	11.92	191.8	124.0
August 7	186.5	13.00	182.5	115.0

Results are expressed as mgm per 100 ml plasma

Case #7: In the case of this patient (UAH #95040) a left nephrectomy was carried out for renal cell carcinoma (hypernephroma). The urologist was of the opinion that the lesion had been completely excised surgically. It was observed that the plasma galactose-mannose level gradually fell to normal (Figure 19) during the three week period of convalescence following his operation. After a sympton-free interval of 24 months the patient died of recurrence of the carcinoma.

Case #8: Serial determination of the plasma glycoproteins were carried out on this patient (UAH #216947), who was admitted on June 3rd and was operated on for adenocarcinoma of the rectum without metastases on June 19th. His convalescence was uneventful and he was discharged on July 26th.

The results recorded in Table XX show the high initial plasma galactose-mannose level which tends to return towards normal levels post-operatively. The plasma hexosamine levels which were significantly

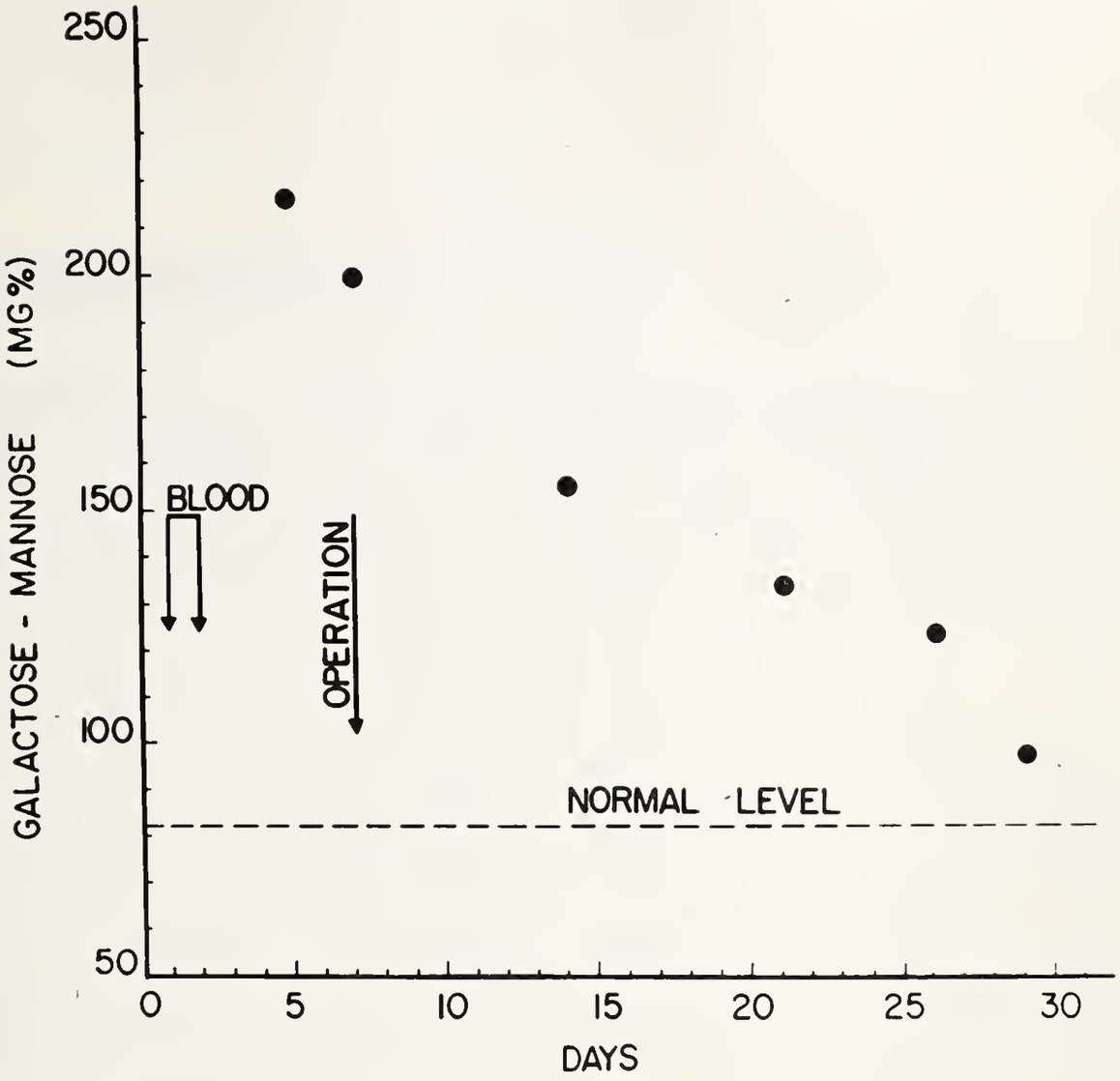


Figure 19

Serial Plasma Galactose-Mannose Levels in a Patient Suffering from Renal Hypernephroma Amenable to Total Surgical Excision

Table XX

Serial Plasma Glycoprotein Levels in a Patient After Successful Treatment of Adenocarcinoma of the Rectum

Date	Galactose-mannose	Fucose	Hexosamine	N-acetylneuraminic acid
June 18	189.0	9.6	117.5	76.8
June 26	159.0	10.8	86.0	89.6
July 3	142.2	10.6	147.8	86.5
July 10	129.8	8.2	147.8	78.6
July 19	143.0	10.2	210.6	82.8
July 24	124.9	10.2	175.0	81.5
August 17	123.8	8.3	109.2	76.8
August 24	124.9	9.3	-	83.6

Results are expressed as mgm per 100 ml plasma

increased during the recovery period also finally began to manifest a trend toward normality. The plasma fucose and N-acetylneuraminic acid showed negligible variations, before and after the operation.

Case #9: Determination of galactose-mannose levels on this patient (UAH #208085) are recorded in Figure 20. After considerable pre-operative preparation he underwent a pelvic exenteration for adenocarcinoma of the rectum invading the bladder. The surgeon was of the opinion that complete removal of the tumour had been accomplished. The gradual return of the galactose-mannose levels towards normal can be seen.

Case #10: This patient (UAH #74836) was admitted on June 6th and underwent abdominoperineal resection for a grade III adenocarcinoma of the rectum without invasion of the lymph nodes on June 20th. Except for a minor urinary tract infection his convalescence was uneventful.

The results in Table XXI show that all carbohydrate constituents of the plasma glycoproteins were elevated before the surgical treatment. However, after a presumeably successful operation on this patient all sugar moieties of plasma, except plasma hexosamine, showed a gradual return to normal level. However, the pattern of return towards normal was not similar for the various plasma protein-bound carbohydrates.

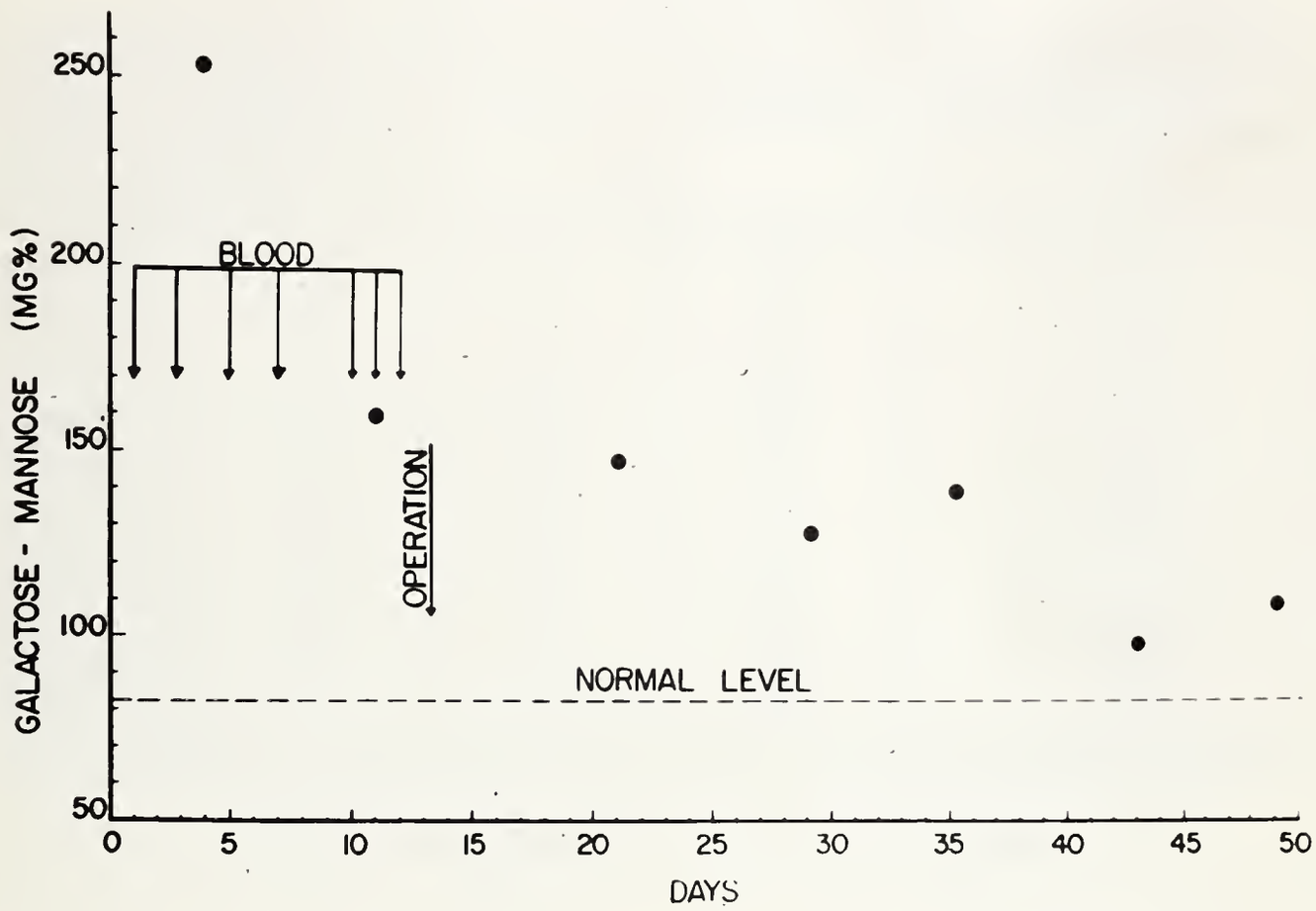


Figure 20

Serial Galactose-Mannose Levels in a Patient Suffering from Rectal Adenocarcinoma Amenable to Total Surgical Excision

Table XXI

Serial Plasma Glycoprotein Levels in a Patient After Successful Treatment of Adenocarcinoma of the Rectum

Date	Galactose-mannose	Fucose	Hexosamine	N-acetylneuraminic acid
June 18	163.0	15.3	132.5	106.7
June 27	179.0	9.6	144.5	115.5
July 4	115.5	11.8	176.8	80.0
July 12	160.1	14.9	183.0	96.5
July 18	153.1	13.5	123.0	88.6
July 25	148.2	7.6	142.8	80.4
August 1	117.2	10.8	185.6	75.8

Results are expressed as mgm per 100 ml plasma

Discussion

It would appear from the results presented above that the various carbohydrate moieties of plasma glycoproteins are elevated in patients suffering from malignant disease and also in certain non-malignant disease states. It was also observed that the magnitude of the increase was not identical for the various protein-bound carbohydrates.

On a basis of these observations it appears unlikely that the determination of plasma glycoproteins of the total plasma proteins will prove to be a useful diagnostic procedure for cancer. It is possible, however, that the changes associated with malignant disease may reside in a specific plasma glycoprotein fraction. This glycoprotein might be present in reduced quantity or even completely absent under normal circumstances. Unfortunately since no attempt has been made to fractionate the plasma proteins in this study this hypothesis has no experimental support at this time. In the present data, however, there is a suggestion that the fucose of the plasma glycoproteins may be elevated exclusively in malignant disease, even including clinically localized carcinoma of the breast.

The data presented would suggest that elevation of hexosamines (glucosamine-galactosamine) in plasma has little disease linked specificity. These carbohydrates were found to be elevated above normal levels in malignant and non-malignant diseases. The variation in the N-acetylneuraminic acid is however of considerable interest. Significant elevation of this carbohydrate above normal was observed in all forms of

malignant disease except localized carcinoma of the breast. However, in the few cases investigated in which carcinoma of the breast had progressed to a stage of proven metastatic involvement significant elevation of protein-bound N-acetylneuraminic acid did occur. This probably merely reflects the greater mass of neoplastic tissue present in those cases with metastases but warrants further exploration in the event that the activity of the metastatic deposits may, in some way, differ from that of the primary lesion.

The intimate relationship of the elevation of the plasma glycoproteins to the malignant process was further substantiated in this study by the return of the abnormally high glycoprotein levels towards normal following assumed total surgical excision of the malignant focus. However, the opposite was true following incomplete surgical removal or unsuccessful X-irradiation or chemotherapy. It is necessary to emphasize again that the magnitude of the elevation above normal or return towards normal levels is not uniform for the different carbohydrate constituents of plasma glycoproteins.

While the clinical implications of elevations in the plasma glycoproteins are of considerable interest the demonstration of the mechanism by which the elevation is accomplished would be of far greater importance.

The present study unfortunately gives no clue to the solution of this problem. It would appear, however, that an approach could be made to this interesting problem by means of serial plasma glycoprotein determinations following induced malignant tumour growth in experimental animals.

B. The effect of the growth of the Walker-256 carcinoma on the rat plasma glycoproteins

The natural history of the progress of implanted Walker-256 carcinoma in our laboratory corresponded to that recorded by Weimer et al (47). After implantation there was an initial period of slow growth, which corresponded to the establishment of the tumour, followed by a period of rapid growth. No metastases were grossly recognizable until the eighth day, at which time the abdominal lymph nodes were enlarged and neoplastic deposits were microscopically manifest. These nodes continued to enlarge during the period of observation and by the twenty-fourth day formed a mass, by coalescence, which measured an average of 6 cm in length. Twelve days after implantation lung metastases became apparent in most of the animals and progressed to the point of partial replacement of the lung by about the twenty-fourth day. If permitted to run its natural course the Walker carcinoma leads to death of the host in from three to four weeks from implantation.

The experimental results are summarized in Table XXII-XXV and Figures 21, 22, 23, 24.

Table XXII shows that the hematocrit level of the tumour bearing animals was significantly reduced by the twelfth day following implantation. The hemoglobin showed some variations from the control group during the first 16 days but became significantly decreased only by the eighteenth day after tumour transplantation. The total plasma protein concentration failed to demonstrate any marked alteration from normal group throughout

the period of study.

Two means of presentation have been utilized for all carbohydrate constituents of plasma glycoproteins. In Tables XXII, XXIV, XXVI and Figures 21, 22, 23, 24 the carbohydrate values are expressed as mgm of carbohydrate per 100 ml plasma. In Tables XXIII, XXV, XXVII and again in Figures 21, 22, 23, 24 they are expressed as gm of carbohydrate per 100 gm of plasma protein.

Changes in the hexose (galactose and mannose) moieties of plasma glycoproteins are depicted in Tables XXII, XXV and Figure 21. The results indicate a slow initial rise which corresponds with the establishment of the tumour. This was followed by a rapid increase in the plasma level of the hexoses corresponding with the period of rapid tumour growth. The elevation is statistically significant by the tenth day after implantation.

Tables XXII-XXV and Figure 22 show the changes in the level of the hexosamines (glucosamine-galactosamine). The effect of tumour growth on the level of these carbohydrate constituents of plasma glycoproteins were not as dramatic as in the case of galactose and mannose. However, statistically significant elevation of hexosamines did occur by the sixteenth day following implantation.

The experimental results in Tables XXII-XXV and Figure 23 show that the plasma N-acetylneuraminic acid levels exhibited a response which is similar to that observed with galactose and mannose. A continuous rise in N-acetylneuraminic acid content begins as early as the third day after implantation and reached a maximal elevation on the eighteenth day.

Table XXV

Effect of Neoplastic Growth on Plasma Protein-Bound Carbohydrates in Rats with Intramuscular Walker-256 Carcinoma

(Rapid tumour growth)

Tumour age (days)	Number of animals	Total plasma protein, #	Galactose-mannose # +	Glucosamine-galactosamine # +	N-acetyl-neuraminic acid, # +	Fucose # +	Total carbo-hydrates # +
Normal	30	6.2 ±0.16	2.3 ±0.04	1.8 ±0.02	1.3 ±0.02	0.16 ±0.01	5.56
10	7	6.3 ±0.14	4.1 ±0.01*	2.2 ±0.10	2.3 ±0.06*	0.17 ±0.01	8.77*
12	7	6.3 ±0.07	3.5 ±0.05	2.1 ±0.09	2.0 ±0.07*	0.17 ±0.01	7.77*
14	8	6.4 ±0.07	4.2 ±0.12*	2.1 ±0.07	2.8 ±0.06*	0.19 ±0.06	9.29*
16	8	5.6 ±0.15	4.7 ±0.16*	2.7 ±0.17*	2.5 ±0.13*	0.18 ±0.01	10.08*
18	8	5.6 ±0.22	4.2 ±0.24*	2.5 ±0.03*	2.9 ±0.09	0.23 ±0.02*	9.83*
20	6	5.2 ±0.14	5.2 ±0.10*	2.4 ±0.19*	2.6 ±0.13*	0.22 ±0.05*	10.42*
22	4	5.1 ±0.20	5.0 ±0.10*	2.5 ±0.08*	2.7 ±0.07*	0.21 ±0.01*	10.41*
24	4	6.0 ±0.40	4.0 ±0.21*	2.1 ±0.11	2.1 ±0.10*	0.20 ±0.01	8.40*

+ = Protein-bound carbohydrate in gm per 100 gm total plasma protein

= Including the standard error of the mean

* = P less than 0.01

Table XXII (cont.)

Effect of Neoplastic Growth on Plasma Protein-Bound Carbohydrates in Rats with Intramuscular Walker-256 Carcinoma

(Rapid tumour growth)

Tumour age (days)	Number of animals	Tumour diameter (cm)	Hemoglobin (gm %) #	Hematocrit (%) #	Galactose-mannose # +	Glucosamine-galactosamine	N-acetyl-neuraminic acid, # +	Fucose # +	Total carbohyd-rates, +
Normal	30		12.6 ±0.28	47.0 ±1.00	144.0 ±2.3	111.9, ±1.2	80.8 ±1.3	10.0 ±0.5	346.7
10	7	3.5-4.5	12.8 ±0.20	40.5 ±0.60	258.0 ±6.2*	141.0 ±6.5*	141.6 ±3.7*	10.6 ±0.6	551.2*
12	7	4.5-5.5	11.9 ±0.25	36.5 ±1.00*	222.3 ±3.3*	134.2 ±5.8	128.7 ±4.3*	10.5 ±0.6	495.7*
14	8	5.0-5.5	10.3 ±0.60	34.0 ±1.20*	268.9 ±7.6*	130.9 ±4.6	176.8 ±3.7*	12.3 ±1.0	588.9*
16	8	5.0-5.5	11.1 ±0.50	31.0 ±0.50*	261.5 ±9.2*	151.4 ±9.3*	142.3 ±7.1*	9.9 ±0.2	565.1*
18	8	5.5-6.0	9.6 ±0.20*	33.5 ±1.50*	236.6 ±13.5*	138.2 ±1.9*	162.2 ±5.5*	12.9 ±1.1	549.9*
20	6	5.5-6.5	8.0 ±1.20	26.5 ±1.20*	271.2 ±5.6*	122.8 ±9.8	135.1 ±7.0*	11.6 ±1.0	540.7*
22	4	6.0-6.5	10.5 ±0.30		253.9 ±5.0*	129.5 ±4.2	135.4 ±3.7*	10.7 ±0.4	529.5*
24	4	6.5-7.0	7.5 ±0.10*	31.0 ±1.60*	241.1 ±12.6*	123.9 ±6.6	122.8 ±6.5*	12.1 ±0.1	499.9*

+ = Protein-bound carbohydrate in mgm per 100 ml plasma

= Including the standard error of the mean

* = P less than 0.01

Table XXIII

Effect of Neoplastic Growth on Plasma Protein-Bound Carbohydrates in Rats with Intramuscular Walker-256 Carcinoma

($low tumour growth)

Tumour age (days)	Number of animals	Total plasma protein ++ #	Galactose-mannose # +	Glucosamine-galactosamine # +	N-acetyl-neuraminic acid, # +	Fucose # +	Total carbo-hydrates # +
Normal	30	6.2 ±0.16	2.3 ±0.04	1.8 ±0.02	1.3 ±0.02	0.16 ±0.01	5.56
1	6	6.0 ±0.07	2.5 ±0.08	2.0 ±0.03	1.6 ±0.03	0.16 ±0.01	6.25
2	6	6.5 ±0.14	2.5 ±0.07	2.0 ±0.04	1.8 ±0.07	0.17 ±0.01	6.47
3	6	5.9 ±0.20	3.2 ±0.08	1.9 ±0.06	2.0 ±0.09*	0.22 ±0.06*	7.32*
4	6	6.2 ±0.22	3.2 ±0.11	1.7 ±0.09	1.7 ±0.05	0.19 ±0.03	6.79
5	6	6.0 ±0.14	3.3 ±0.03	2.1 ±0.05	2.0 ±0.03*	0.17 ±0.02	7.57*
6	6	6.5 ±0.14	3.2 ±0.09	1.6 ±0.05	1.9 ±0.11	0.17 ±0.01	6.87
8	6	6.6 ±0.07	3.2 ±0.03	1.5 ±0.07	1.8 ±0.07	0.17 ±0.01	6.67

\+ = Protein-bound carbohydrate in gm per 100 gm total plasma protein

\# = Including the standard error of the mean

* = P less than 0.01

++ = Total plasma protein in gm per 100 ml plasma

Table XXIII (cont.)

Effect of Neoplastic Growth on Plasma Protein-Bound Carbohydrates in Rats with Intramuscular Walker-256 Carcinoma

(Rapid tumour growth)

Tumour age (days)	Number of animals	Total plasma protein # ++	Galactose-mannose # +	Glucosamine-galactosamine # +	N-acetyl-neuraminic acid, # +	Fucose # +	Total carbo-hydrates # +
Normal	30	6.2 ±0.16	2.3 ±0.04	1.8 ±0.02	1.3 ±0.02	0.16 ±0.01	5.56
10	7	6.3 ±0.14	4.1 ±0.01*	2.2 ±0.10	2.3 ±0.06*	0.17 ±0.01	8.77*
12	7	6.3 ±0.07	3.5 ±0.05	2.1 ±0.09	2.0 ±0.07*	0.17 ±0.01	7.77*
14	8	6.4 ±0.07	4.2 ±0.12*	2.1 ±0.07	2.8 ±0.06*	0.19 ±0.06	9.29*
16	8	5.6 ±0.15	4.7 ±0.16*	2.7 ±0.17*	2.5 ±0.13*	0.18 ±0.01	10.08*
18	8	5.6 ±0.22	4.2 ±0.24*	2.5 ±0.03*	2.9 ±0.09	0.23 ±0.02*	9.83*
20	6	5.2 ±0.14	5.2 ±0.10*	2.4 ±0.19*	2.6 ±0.13*	0.22 ±0.05*	10.42*
22	4	5.1 ±0.20	5.0 ±0.10*	2.5 ±0.08*	2.7 ±0.07*	0.21 ±0.01*	10.41*
24	4	6.0 ±0.40	4.0 ±0.21*	2.1 ±0.11	2.1 ±0.10*	0.20 ±0.01	8.40*

\+ = Protein-bound carbohydrate in gm per 100 gm total plasma protein

\# = Including the standard error of the mean

* = P less than 0.01

++ = Total plasma protein in gm per 100 ml plasma

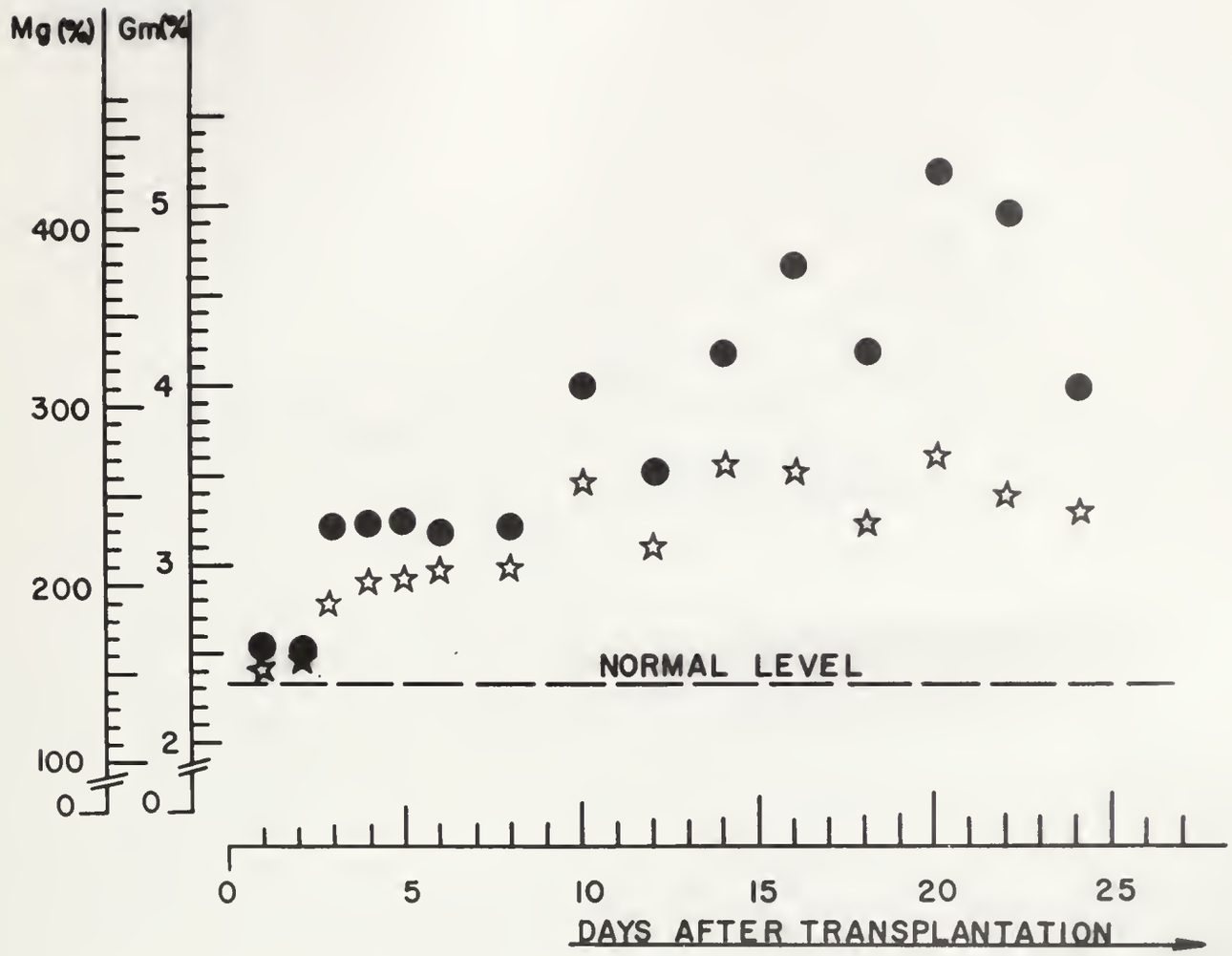


Figure 21

Plasma Galactose-Mannose Levels in the Rat Following the Implantation of Walker-256 Carcinoma

Circles = gm of galactose-mannose per 100 gm plasma protein
Stars = mgm of galactose-mannose per 100 ml plasma

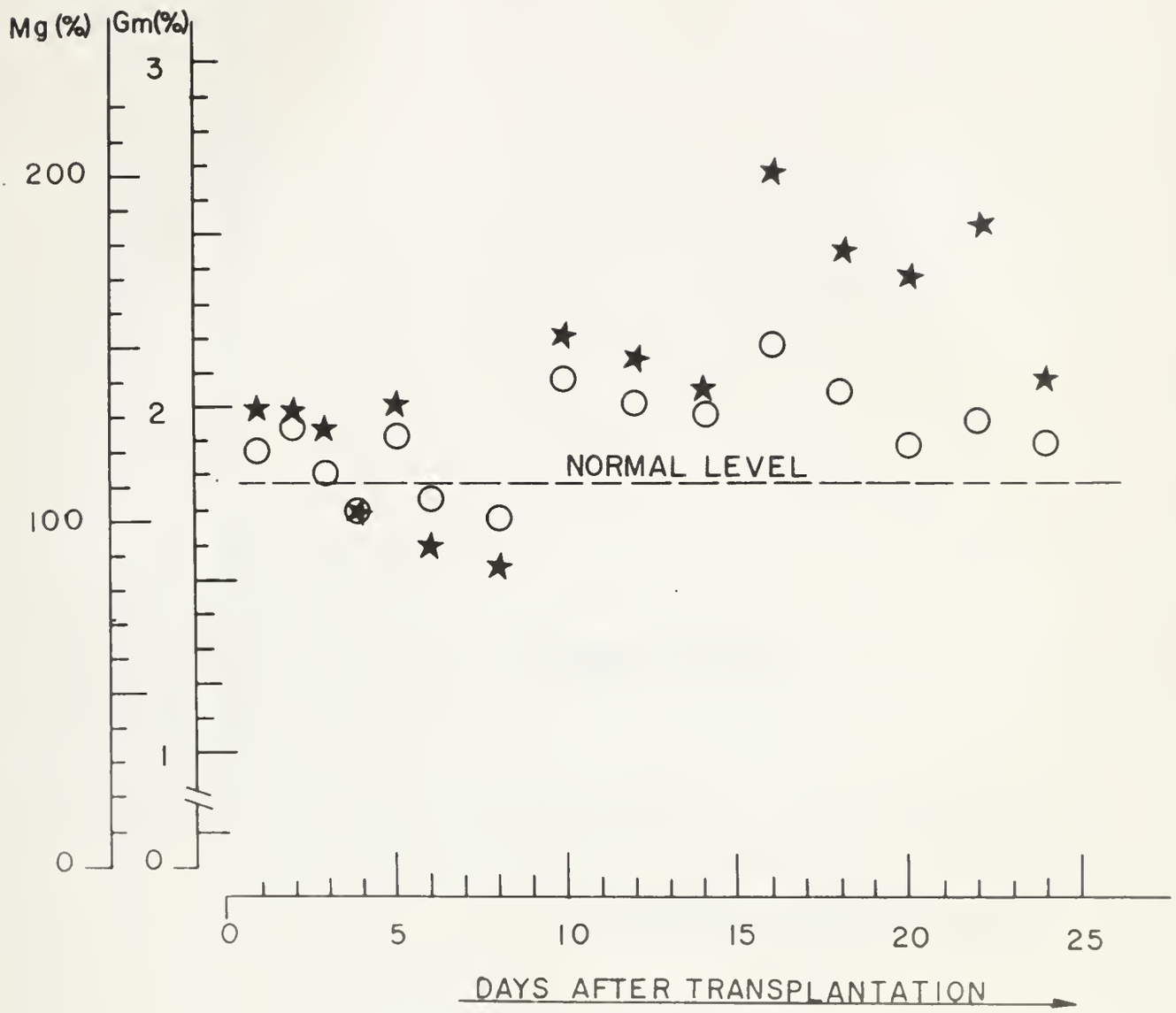


Figure 22

Plasma Glucosamine-Galactosamine Levels in the Rat Following the Implantation of Walker-256 Carcinoma

Stars = gm of glucosamine-galactosamine per 100 gm plasma protein
Circles = mgm of glucosamine-galactosamine per 100 ml plasma

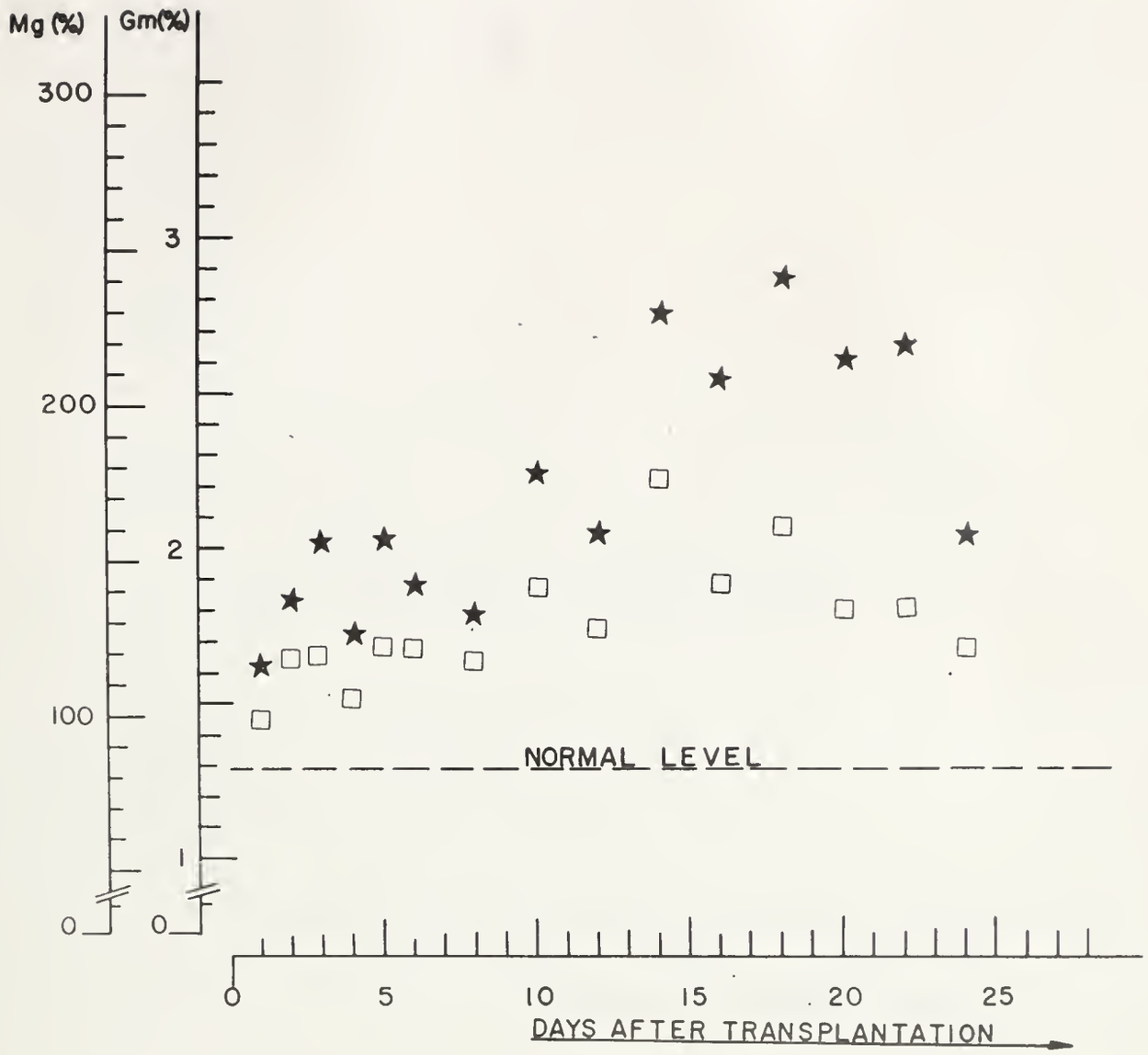


Figure 23

Plasma N-acetylneuraminic Acid Level in the Rat Following the Implantation of Walker-256 Carcinoma

Stars = gm of N-acetylneuraminic acid per 100 gm plasma protein
Squares = mgm of N-acetylneuraminic acid per 100 ml plasma

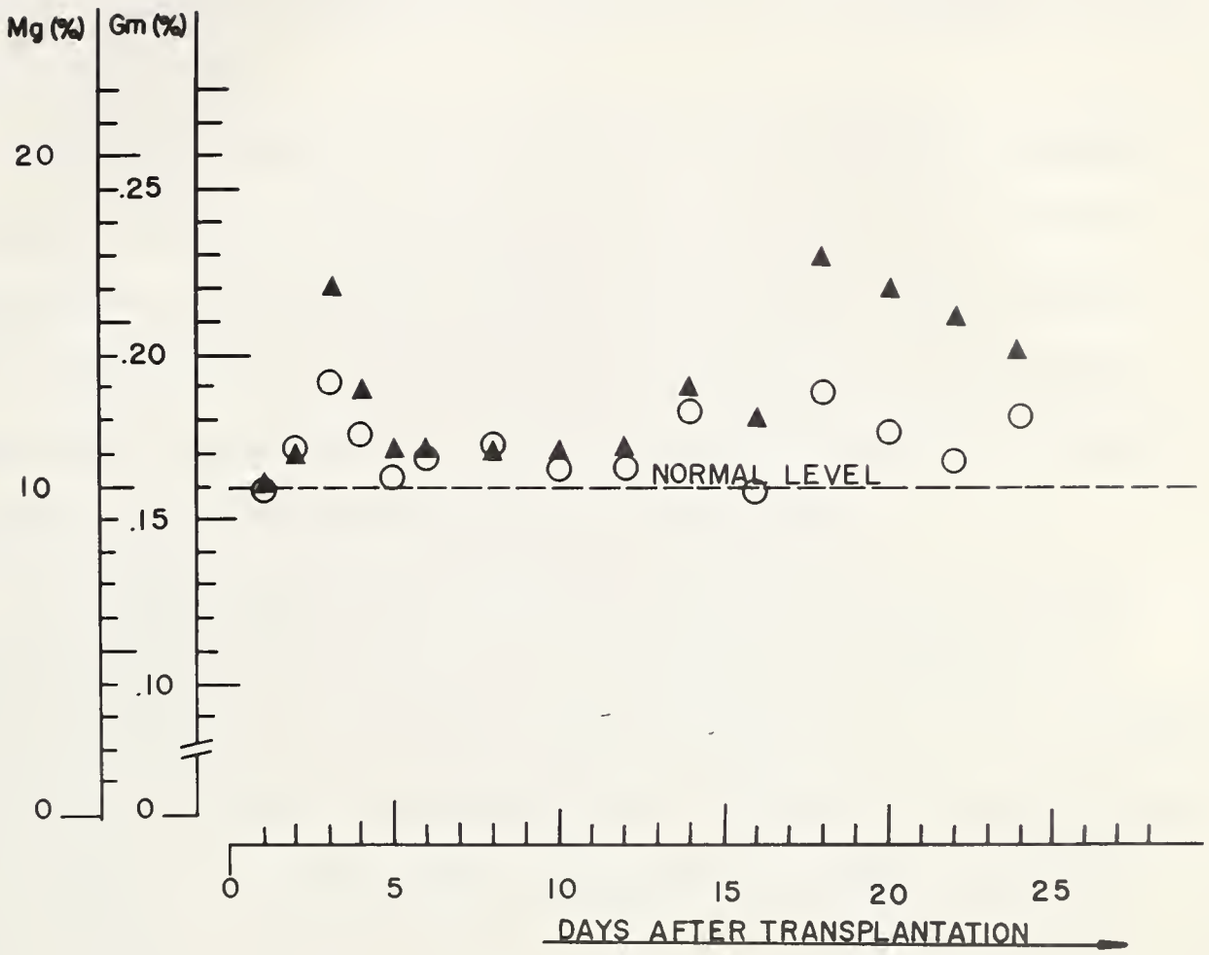


Figure 24

Plasma Fucose Level in the Rat Following the Implantation of Walker-256 Carcinoma

Triangles = gm of fucose per 100 gm plasma protein
Circles = mgm of fucose per 100 ml plasma

A significant elevation in fucose was apparent on the third day after tumour implantation, followed by a return to normal levels. Finally a sustained elevation was observed from the eighteenth to the twenty-fourth days.

The total concentration of all the above carbohydrate moieties of plasma glycoproteins showed significant elevations. The maximal concentration of glycoprotein carbohydrate in 100 ml plasma appared on the fourteenth day after implantation. The ratio of total plasma protein-bound carbohydrate to total plasma protein between fourteenth and twenty-second day after implantation was 1 to 10.

The control experiment on 32 rats, which were injected intramuscularly with an homologous muscle homogenate, is summarized in Tables XXVI, XXVII. The results indicate that the total plasma protein and galactose-mannose, glucosamine-galactosamine and N-acetylneuraminic acid levels of the plasma glycoproteins did not exhibit significant variations from the control group. Fucose alone exhibited levels on the third and fourth day following intramuscular implantation of muscle homogenate, which were significantly elevated as compared with control values.

Table XXIV

Effect of Intramuscular Injection of Homogenous Muscle on Plasma Protein-Bound Carbohydrate in the Rat

Tumour age (days)	Number of animals	Galactose mannose # +	Glucosamine-galactosamine # +	N-acetyl-neuraminic acid, # +	Fucose # +	Total carbo-hydrates # +
Normal	8	138.8 ±3.9	130.6 ±4.8	90.0 ±3.4	10.1 ±0.3	370.4
1	4	121.3 ±2.9	113.8 ±3.8	87.1 ±1.1	11.9 ±0.6	334.1
2	4	129.5 ±3.3	109.9 ±0.4	86.1 ±1.0	11.4 ±0.5	336.9
3	4	140.6 ±4.5	134.6 ±1.8	96.1 ±2.9	11.7 ±0.3	383.0
4	4	143.9 ±4.7	147.3 ±6.7	99.3 ±6.2	13.7 ±0.6*	404.2
5	4	162.5 ±3.9	129.1 ±6.0	102.6 ±1.3	10.0 ±0.5	404.2
6	4	143.3 ±2.2	136.4 ±2.6	95.8 ±0.9	12.8 ±0.4	388.3
8	4	157.1 ±4.7	135.6 ±6.9	111.2 ±5.2	10.0 ±0.9	413.9
14	4	155.3 ±3.0	132.6 ±5.5	101.0 ±3.7	10.4 ±1.1	399.3

+ = Protein-bound carbohydrate in mgm per 100 ml plasma

= Including the standard error of the mean

* = P less than 0.01

Table XXV

Effect of Intramuscular Injection of Homogenous Muscle on Plasma Protein-Bound Carbohydrate in the Rat

Tumour age (days)	Number of animals	Total plasma protein # ++	Galactose-mannose # +	Glucosamine-galactosamine # +	N-acetyl-neuraminic acid, # +	Fucose # +	Total carbo-hydrates # +
Normal	8	5.76 ±0.2	2.41 ±0.07	2.27 ±0.03	1.58 ±0.06	0.18 ±0.003	6.44
1	4	5.60 ±0.6	2.19 ±0.05	2.03 ±0.07	1.55 ±0.02	0.21 ±0.001	5.98
2	4	5.80 ±0.6	2.24 ±0.06	1.90 ±0.01	1.49 ±0.02	0.20 ±0.009	5.83
3	4	5.50 ±0.7	2.54 ±0.08	2.43 ±0.03	1.73 ±0.05	0.21 ±0.002	6.91
4	4	5.60 ±0.7	2.59 ±0.08	2.64 ±0.11	1.78 ±0.11	0.24 ±0.010*	7.25
5	4	5.92 ±0.4	2.68 ±0.06	2.13 ±0.10	1.69 ±0.02	0.17 ±0.010	6.67
6	4	5.20 ±0.9	2.78 ±0.05	2.64 ±0.05	1.86 ±0.02	0.25 ±0.009	7.53
8	4	5.70 ±0.5	2.76 ±0.08	2.41 ±0.12	1.95 ±0.09	0.17 ±0.002	7.29
14	4	5.30 ±0.8	2.63 ±0.06	2.24 ±0.10	1.91 ±0.07	0.20 ±0.020	6.98

\+ = Protein-bound carbohydrate in gm per 100 gm total plasma protein

\# = Including the standard error of the mean

* = P less than 0.01

++ = Total plasma protein in gm per 100 ml plasma

Discussion

The growth of Walker-256 carcinoma in the Sprague-Dawley rat following implantation is associated with significant increases in the six carbohydrate constituents of the plasma glycoproteins. It was observed moreover, that the galactose-mannose and N-acetyl-neuraminic acid components of plasma glycoproteins were the first to show elevation after tumour implantation. The levels of these carbo-hydrates became significantly and consistently elevated by the tenth day following implantation. The early changes in the level of galactose-mannose and N-acetylneuraminic acid may well be non-specific. It is clear from the above experimental results however that the elevation observed after fifth day is intimately related to the neoplastic process.

The significant elevation of hexosamines which occurs about sixteen days after implantation is less pronounced than with the galactose-mannose and N-acetylneuraminic acid. Except for the early non-specific increase on the third and fourth days the elevation of plasma fucose is the last event to occur, on about the eighteenth day. In addition the magnitude of its maximal elevation is about half that observed with the other carbohydrate constituents.

A survey of the available publications dealing with experimental host-tumour systems in animals gives no basis for comparison, except in the case of protein-bound galactose-mannose. However, the increase of galactose-mannose in the present study is much greater than the elevation reported by Shetlar et al (45) which occurred at the end

of the fifth week post implantation in their study. However Shetlar et al (45) used subdermal implantation of Walker-256 carcinoma in Sprague-Dawley rats and it has been demonstrated (47) that the site of implantation is of extreme importance with respect to the magnitude of the plasma protein-bound galactose-mannose response. Baldwig (46) using sarcoma-66 in Wistar rats and Weimer et al (47) using Walker-256 carcinoma in Sprague-Dawley rats reported maximal elevation on the fourteenth day, but the magnitude of the increase they obtained was less marked than in the experiments reported here.

It has been demonstrated by Shetlar et al (44, 45, 105) and Miettinen (106) that elevations of galactose-mannose levels occurred following experimentally induced inflammation and after physical stress. It appeared questionable, in this study whether a single intramuscular injection (during the tumour transplantation) would be sufficient to initiate an inflammation and consequently would cause an alteration in the plasma glycoproteins. Clinical inflammation of the implanted limb did not occur in this study. It is apparent from our double control study that a significant elevation of galactose-mannose, glucosamine-galactosamine and N-acetylneuraminic acid did not occur during the 14 days observation period following implantation of an homologous muscle implant. However, the early increase in plasma fucose level which occurred after the injection of muscle homogenate, corresponded to the response after the transplantation of tumour cells, which would indicate that at least in the case of fucose this elevation is likely to be non-specific in nature.

These experimental results, like the study on human plasma glycoproteins, indicate a definite relationship between the elevated plasma glycoproteins and the natural development of the tumour. Although elevation in each carbohydrate constituent was demonstrated, the intervals between implantation and the occurrence of a significant increase varied for each carbohydrate. This observation would suggest that the site or sites of formation of the various carbohydrate moieties of the elevated plasma glycoproteins may be mediated by different mechanism. This hypothesis, as far as can be determined, has not previously been put forward, nor is any other experimental support for it available.

It is apparent that the estimation of total plasma glycoproteins in rats with Walker-256 carcinoma gave general but limited information regarding the effect of tumour growth on plasma glycoproteins. However, the way is prepared for further investigations concerning the chemistry, the site or sites of formation and the physiological significance of the elevated plasma glycoproteins. Further investigations will be necessary to reveal the physiological and biological importance of plasma glycoproteins, to investigate the relation of plasma glycoprotein to the connective and other tissue glycoproteins, and to study the synthesis and release of glycoproteins by utilizing modern research techniques, including the use of radioactive tracer compounds.

VI. BIBLIOGRAPHY

1. Freund, E.: Zentr. Physiol., 6:345 (1892).
2. Morner, K.A.H.: Zentr. Physiol., 7:581 (1893).
3. Zanetti, C.: Ann. di Chim. e di Farmac, 12:1 (1897).
4. Zanetti, C.: Gazz. chim. ital., 33:160 (1903).
5. Bywaters, W.: Biochem. Z., 15:322 (1909).
6. Grevenstuck, A.: Erg. d. Ges. Physiol., 37:281 (1929).
7. Lustig, B. and Langer, A.: Biochem. Z., 242:320 (1931).
8. Rimington, C.: Biochem. J., 25:1062 (1931).
9. Rimington, C.: Erg. d. Ges. Physiol., 35:712 (1933).
10. Partridge, S.M.: Nature, 158:270 (1946).
11. Partridge, S.M.: Biochem. J., 42:238 (1948).
12. Dische, Z. and Shettles, L.B.: J. Biol. Chem., 175:595 (1948).
13. Waldron, D.M.: Nature, 170:461 (1952).
14. Winzler, R.J.: Methods of Biochem. Anal., Vol. II:279 (1955).
15. Odin, L.: Acta Chem. Scand., 9:714 (1955).
16. Goa, J.: Scand. J. Clin. Lab. Invest., 7.Suppl., 22:1 (1955).
17. Schultze, H.E.: Scand. J. Clin. Lab. Invest. Vol. 10, suppl. 32:135 (1957).
18. Schultze, H.E.: Deut. Med. Wochschr., 83:1742 (1958).
19. Schultze, H.E.; Heide, K. and Haupt: Klinische Wochenschrift, 40:729 (1962).
20. Bettelheim-Jevons, F.R.: Advances in Protein Chemistry, 13:35 (1958).
21. Winzler, R.J.: Glycoproteins of plasma in Ciba Foundation Symposium on the Chemistry and Biology of Mucopolysaccharides, p. 245, Ed. Wolstenholm, G.E.W. and O'Connor, M. Boston, Little, Brown and Co., 1958.

22. Winzler, R.J.: Glycoproteins in the plasma proteins, Vol.I., p. 309. ed. Putnam, F.W., New York, Academic Press Inc. (1960).

23. Biserte, G.: Bull. soc. chim. biol. 39., suppl. III. 93. (1957).

24. Laurent, B.: Scand. J. Clin. Invest. 10 suppl. 32:9 (1958).

25. Stary, Z.: Erg. der Physiol., 50:174 (1959).

26. Bostrom, H., Roden, L. and Yamashina, I.: J. Biol. Chem., 230:381 (1959).

27. Spiro, R.G.: J. Biol. Chem., 234:742 (1959).

28. Almquist, P.Q. and Lausing, E.: Scand. J. Clin. Lab. Invest., 9:179 (1957).

29. Butler, L.O.: Brit. J. Cancer, 5:225 (1951).

30. Galletti, F.: Biochemica, Fisiologia e. Patologia Bologna Medica, ed. Gastone Gelli (1958).

31. Kellen, J.: Biochemie, Klinik and Lab.-diagnostik, Unter Mitarbeit von J. Musil Leipzig, G. Thieme, (1960).

32. Riegal, G., Jones, R., Welch, K.J. and Valantine, J.: Cancer Research, 11:453 (1951).

33. Seibert, F.B., Pfaff, M.L. and Seibert, M.V.: Arch. Biochem., 18:279 (1948).

34. Seibert, F.B., Seibert, M.V., Atno, A.J. and Combell, H.W.: J. Clin. Invest. 26:90 (1947).

35. Shetlar, M.R., Shetlar, C.L., Richmond, V. and Everett, M.R.: Cancer Research, 10:681 (1950).

36. Cohen, R.J. and Byham, B.: J. Lab. Clin. Med., 35:869 (1950).

37. Koiw, E. and Gronwall, A.: J. Clin. Lab. Invest., 4:244 (1952).

38. Berkman, J., Rifkin, H. and Ross, G.: J. Clin. Invest., 32:415 (1953).

39. Kelley, V.C.: J. Pediatrics, 40:405 (1952).

40. Shetlar, M.R., Foster, J.V., Kelly, K.H., Shetlar, C.L., Bryan, R.S. and Everett, M.R.: Cancer Research, 9:515 (1949).

41. Bohm, P.: Klinisch-chemische und biologische uber die Neuraminsaure in Korperflussigkeiten, Universitat Bonn (1958).

42. Saifer, A., Aronson, S.M., Zymaris, M.C. and Volk, B.W.: Proc. Soc. Exp. Biol. Med., 91:394 (1956).

43. Saifer, A., Volk, B.W. and Aronson, S.M.: J. Dis. Childh., 97:745 (1959).

44. Catchpole, H.R.: Proc. Soc. Exp. Biol. Med., 75:221 (1950).

45. Shetlar, M.R., Erwin, C.P. and Everett, M.R.: Cancer Research 10:445 (1950).

46. Baldwin, R.W. and Harries, H.J.: Brit. J. Cancer, 12:99 (1958).

47. Weimer, H.E., Quin, F.A., Redlich-Moshin, J. and Nishihara, H.: J. Nat. Cancer Inst., 19:409 (1957).

48. Heiskell, C.L., Carpenter, C.M., Weimer, H.E. and Nakagawa, S.: Ann. New York Ac. of Sciences, 94:183 (1961).

49. Bollet, A.J., Rozhin, J. and Simpson, W.: J. Clin. Invest., 36:51 (1957).

50. Dische, Z. and Osnos, M.: Fed. Proc., 9:165 (1950).

51. Bonomo, E. and Bonnelli, M.: Tumori, 38:351 (1952).

52. Mecroff, M.: Sem. Med. (B. Aires), 113:60 (1958).

53. Meyer, K.: Some Conjugated Proteins, W.H. Cole, ed. Rutgers, University Press, p. 63, 1953.

54. Committee on Protein Nomenclature, J. Biol. Chem., 4:xlviii (1908).

55. Rimington, C.: Biochem. J., 34:931 (1940).

56. Weimer, H.E., Mehl. J.W. and Winzler, R.J.: J. Biol. Chem., 185:569 (1950).

57. Schmid, K.: J. Am. Chem. Soc., 75:2532 (1953).

58. Schmid, K.: J. Am. Chem. Soc., 75:60 (1953).

59. Schmid, K.: J. Am. Chem. Soc., 77:742 (1955).

60. Masamune, H.: Proc. 3rd Intern. Congr. Biochem., Brussels, p. 72 (1955).

61. Gottschalk, A.: Prospectives in Biology and Medicine, Vol. V, No. 3:327 (1962).

62. Dische, Z.: Biochem. Z., 201:74 (1928).

63. Bierry, H.: Ber. u. d. ges. Physiol., 52:607 (1930).

64. Bierry, H.: Compt. rend., 116:702 (1934).

65. Kalckar, H.M. and Maxwell, E.S.: Physiol. Revs., 38:77 (1958).

66. Kalckar, H.M.: Advances in Enzymology, 20:111 (1958).

67. Cabib, E. and Leloir, L.F.: J. Biol. Chem., 206:779 (1954).

68. Shetlar, M.R.: Ann. New York Ac. of Sciences, 94:44 (1961).

69. Roseman, S.: Ann. Rev. Biochem., 28:545 (1959).

70. Pogell, B.M.: Biochem. biophysica Acta, 21:205 (1956).

71. Brown, D.H.: Biochem. biophysica Acta, 16:429 (1955).

72. Davidson, E.A.: J. Biol. Chem., 226:125 (1957).

73. Brown, D.H.: J. Biol. Chem., 204:877 (1953).

74. Hurlbert, R.B. and Potter, V.R.: J. Biol. Chem., 209:1 (1954).

75. Strominger, J.L.: Symposium on Connective Tissue, 1962 New York.

76. Blix, G.: Uber die Kohlenhydratgruppen des Submaxillarismucins, Hoppe-Seyl. Z., 240:43 (1936).

77. Blix, F.G., Gottschalk, A. and Klenk, E.: Nature, 179:1088 (1957).

78. Gottschalk, A.: Arch. Biochem. Biophys., 59:39 (1957).

79. Comb, D.G. and Roseman, S.: J. Am. Chem. Soc., 80:497 (1958).

80. Warren, L. and Blacklow, R.S.: Biochem. and Biophys. Res. Comm. 7:433 (1962).

81. Werner, I. and Odin, L.: Acta soc. Med. Upsalien, 57:230 (1952).

82. Roseman, S., Jourdian, G.W., Watson, D. and Rood, R.: Proc. Natl. Acad. Sci., U.S. 47:958 (1961).

83. Warren, L. and Felsenfeld, H.: J. Biol. Chem., 237:1421 (1962).

84. Warren, L. and Blacklow, R.S.: J. Biol. Chem., 237:3527 (1962).

85. Brunetti, P., Jourdian, G.W. and Roseman, S.: J. Biol. Chem., 237:2447 (1962).

86. Gottschalk, A.: The Chemistry and Biology of Sialic acids and Related Substances, Cambridge, University Press, (1960).

87. Spinger, G.F. and Williamson, P.: Biochem. J., 85:282 (1962).

88. Ginsburg, V. and Kirkman, H.N.: J. Am. Chem. Soc. 80:3481 (1958).

89. Ginsburg, V.: J. Am. Chem. Soc., 80:4426 (1958).

90. Kowalewski, K.: Proc. Soc. Exp. Biol. Med., 120:448 (1959).

91. Hainline, A.: Standard methods of clinical chemistry, Vol. II, p. 49, ed. Seligson, D., New York Ac. Press Inc. 1958.

92. Natelson, L.: Am. J. Clin. Path., 21:1153 (1951).

93. Wolfson, W.Q. and Cohn, C.: Ann. Arbour, Overbeck Co., 1951.

94. Tillmans, J. and Phillippi, K.: Biochem. Z., 215:36 (1929).

95. Weimer, H.E. and Moshin, J.R.: Am. Rev. Tuberculosis, 68:594 (1952).

96. Elson, L.A. and Morgan, W.T.: Biochem. J., 27:1824 (1933).

97. Boas, N.F.: J. Biol. Chem., 204:553 (1953).

98. Schloss, B.: Anal. Chem., 23:1321 (1951).

99. Svennerholm, L.: Biochimica et Biophysica Acta, 24:604 (1957).

100. Svennerholm, L.: Acta Chemica Scand., 12:547 (1958).

101. Martenssen, E., Raal, A. and Svennerholm, L.: Biochimica et Biophysica Acta, 30:124 (1958).

102. Dische, Z.: J. Biol. Chem., 181:379 (1949).

103. Dische, Z.: Methods of Biochemical Analysis, Vol. II, p. 313, ed. D. Glick Interscience Publisher Inc., New York (1955).

104. Neroth, F.H.: Advances in carbohydrate chemistry, 6:83, ed. C.S. Hudson and S.M. Cantor Academic Press, New York, 1951.

105. Shetlar, M.R., Bryan, P.S., Foster, J.V., Shetlar, C.L. and Everett, M.R.: Proc. Soc. Exp. Biol. Med., 72:294 (1949).

106. Miettinen, T.: Scand. J. Clin. Lab. Invest., 13 (suppl. 61) 1961.